ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

FISCAL YEAR 1971

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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U.S. NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Fiscal Year 1971

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ANNUAL REPORT OF PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
JULY 1, 1970 THROUGH JUNE 30, 1971

The National Institute of Allergy and Infectious Diseases conducts and supports a broad program of research directed at helping medical science find improved ways of diagnosing, treating, and preventing allergic conditions and infectious diseases. In carrying out its mission the Institute continues its role of training future medical research workers.

The reorganization of the Institute effected last year placed both the collaborative program and the intramural program under the Scientific Director. He now has more effective oversight of the contract programs and the intramural programs which has resulted in better coordination of direct Institute programs.

Dr. William I. Gay assumed the position of Associate Director for Extramural Programs on December 1, replacing Dr. Francis Abinanti who returned to personal research and was detailed to Scripps Clinic and Research Foundation, La Jolla, California. The extramural program management has undergone a careful review to improve the mechanism for selection of programs and the management of training grants.

Last year the Institute decided that the scientific base of knowledge of allergic diseases was sufficiently advanced to give promise of the fruitful application of preventive and therapeutic procedures within a few years. The plan was to establish a number of Allergic Disease Centers where clinical aspects of allergic diseases could be studied in conjunction with high quality immunologic research. The studies would be directed at immediate hypersensitivities such as asthma, hay fever, and food allergies.

This year the Institute is establishing Allergic Disease Centers at the following seven institutions: Robert B. Brigham Hospital, Boston; National Jewish Hospital and Research Center and the Children's Asthma Research Institute and Hospital, both of Denver; Washington University, St. Louis; Johns Hopkins University, Baltimore; University of Wisconsin, Madison; and the Scripps Clinic and Research Foundation, La Jolla, California.

To meet the need for data on the prevalence and cost of allergic diseases, a statistical function, under Mr. Joseph Schachter, was established in the Office of the Director. During its first year, the statistical staff: (1) undertook a comprehensive survey of all published and unpublished data sources on allergies, including a detailed review and evaluation of the respective forms and procedures employed; (2) developed the scope of work for a contract to produce uniform, broadly acceptable criteria for the diagnosis of allergies; (3) prepared preliminary national estimates of the prevalence of selected allergies, and of the annual dollar cost of patient visits for these diseases;

(4) prepared recommendations for the Ninth Revision of the International Statistical Classification of Diseases and (5) provided statistical consultation to NIAID staff.

Institute activity in scientific communications is a relevant part of research support. Most significant of these activities are the scientific manuscripts reporting on the research investigations supported by NIAID. During FY 71 approximately 310 manuscripts and research notes stemmed from the intramural staff of the Institute, approximately 1250 were prepared by grantees and 300 by contractors. Another 100 papers were prepared by NIAID staff members for presentation at scientific meetings.

Technical reports, the bulk of which are annual reports from research contractors, were sent to the Department of Commerce "National Technical Information Service" from where the reports are available to the scientific community and the public. A monograph "Primate Malarias," authored by members and ex-members of the Institute (Drs. Coatney, Collins, Warren and Contacos) was prepared for publication by the Government Printing Office. The "Internal Scientific Exchange Memorandum". for intra-Institute dissemination of summaries and abstracts of new manuscripts and meeting abstracts from NIAID staff, was distributed on a semi-monthly basis.

Progress was attained in the NIAID feasibility project for the development of a means for fast and informal scientific communications between research investigators. The "Scientific Memoranda" provide for such scientist-to-scientist communications among the active investigators on a world-wide basis. In this project a participant must contribute communications to the "Memoranda" to remain eligible to receive it. This developmental project has been confined to scientific areas in which a relatively small group of investigators is involved. As of May 1, 1971, the number of participants were: "Interferon Scientific Memoranda" -335; "Leprosy Scientific Memoranda" - 64; "Hepatitis Scientific Memoranda" - 152. Annexed once a year to the ISM is an Interferon Bibliography composed of a MEDLAR printout from the National Library of Medicine. The LSM and the HSM contain a "Continuing Bibliography" composed of "Bibliographic Notices" contributed by the participants each time a manuscript is published.

The funding of scientific meetings is undertaken as a direct responsibility whenever the meetings are a part of NIAID program activities. The contract and grant mechanisms are used to provide support for scientific meetings that are planned and conducted by non-Federal groups. To provide an opportunity for early evaluation of NIAID program relatedness of such meetings, and better coordination of the planning of meetings, the Institute established in November, 1970 the Advisory Group on Scientific Communications (AGSC).

Individuals or organizations seeking support for a scientific meeting are advised on NIAID program interest in the proposed meeting, and accordingly to submit a contract proposal, a grant application, or to seek support elsewhere. A requirement common to all NIAID meeting support projects obliges the planners to prepare promptly for publication in a scientific journal a summary report of the scientific discussions.

The NIAID has cooperated with the Fogarty International Center, NIH, and the Office for International Health, DHEW, in activities that result from formal scientific exchange agreements between the United States and other countries. By far, the largest activity has been under the U.S.-Japan Cooperative Medical Science Program, which is described in another part of this report. Under the "U.S.A.-U.S.S.R. Exchange Agreement" a U.S. Scientific Delegation of six scientists visited institutions in the U.S.S.R. for a period of two weeks beginning August 15, 1970 with special interest in transplantation, transplantation immunology and related subjects. Another U.S. group of three scientists interested in interferon and other antiviral substances visited the U.S.S.R. for a two week period beginning June 15, 1971. Under the "U.S.-Franco Exchange Agreement," the NIAID made arrangements for a visiting scientist from the Centre de Transfusion Sanguine de Lyon to spend three months at Duke University, participating in collaboration and scientific exchanges in the field of histocompatibility research.

The Research Information Section has maintained an effective level of public information activity despite restricted resources during the past three years. Emphasis was placed on interpreting the Institute's role in research on allergic disease and clinical immunology as well as on alerting the press to newsworthy areas concerning infectious diseases.

The Section -- in collaboration with the American Academy of Allergy and the Johns Hopkins Medical Institutions -- sponsored its first science Writers' Seminar in Allergic Diseases January 25 - 26, 1971. Held in Baltimore, the writers heard presentations by ten scientists on basic immunology and several clinical allergy topics, including hayfever, asthma, farmer's lung disease and drug allergy.

A seminar on clinical immunology, arranged by the Section's staff, was held on May 1 and 2 in Atlantic City. It was based on the Institute-sponsored scientific meeting at Brook Lodge, Michigan, dealing with immunologic intervention. Nine scientists synopsized the Brook Lodge meeting, discussing a variety of topics including thymus and bone marrow grants, transfer factor, removal of antigens and other molecules by means of immunoadsorbents, and cancer immunology. An abridged version of the briefing has been prepared as a resource for science writers.

For the third consecutive year, the Section arranged for a press briefing in conjection with the annual meeting for the Institute's vaccine development contracts. The briefing covered four research areas: pneumococcal pneumonia vaccine program, rubella vaccines, mycoplasma vaccines and cholera research.

The Section also arranged for a press orientation and tour of the NIAID-AEC Molecular Anatomy Laboratory in Rockville, Maryland. The briefing emphasized the Institute's utilization of the laboratory and its efforts in hepatitis research, and the tour described how the laboratory purifies, concentrates and identifies viral, bacterial and other subcellular particles.

The Institute started distributing three of its new publications--"Bacteria, the Littlest Cells" and "Viruses, on the Border of Life," and a companion leaflet, "Selected Reading in Microbiology." "Bacteria, the Littlest Cells" won third place for popular publications (in 2 or 3 colors) in the Federal Editors Association annual competition. Research fact sheets continued to be prepared: New issuances covered cholera, mycoplasma and leishmaniasis; revised fact sheets included the common cold, fungal infections, influenza, malaria, rabies, viral hepatitis, and sarcoidosis. The series has 15 publications.

The Interagency Agreement with the Technical Analysis Division of the National Bureau of Standards will terminate with the end of the Fiscal Year 1971. This agreement supported the Project SOAP task group in its systems analysis study of decision making processes and methods for allocation of resources.

The main activities of the past year have been concerned with an attempt, by interview and rating exercises, to discover what relevance means to different people in different contexts. That is, how is scientific, program and health relevance envisaged and judged by people trained as scientists who have different responsible roles in the system of science. A report of this work will be issued in July 1971.

Subsidiary activities have been involved (a) with methods for assessing the socio-economic impact of disease (a report of an 8 month part-time study was issued in December 1970); (b) assessing uniformity of Study Section judgment of scientific merit (a staff paper on the latter, based on one Study Section, was issued March 15, 1970); and (c) assessing the equality of Study Section judgment (several staff studies commenting on methods for standardizing or normalizing priority scores have been completed and informally presented). The latter subject has lost its innovative quality because of the decision to adopt NIH-wide a system for normalizing/standardizing Study Section priority scores.

An account of the origins and activities of the Project SOAP has been presented at briefings to the Executive Officers and B/I/D Directors of NIH, as well as the National Advisory Allergy and Infectious Diseases Council. As a result of the latter briefing the Council has recommended that the Institute begin to develop operational methods of allocating resources based upon program importance and relevance.

GEOGRAPHIC MEDICINE BRANCH

1. SUMMARY

Following dissolution of the Office of International Research (OIR) and transfer of three OIR programs to the NIAID on 1 July 1968, the Geographic Medicine Branch was created in NIAID to manage these programs: The United States-Japan Cooperative Medical Science Program, the International Centers for Medical Research and Training (ICMRT), and the International Research Career Development Program (IRCDP). The IRCDP has since become inactive.

I. The United States-Japan Cooperative Medical Science Program

Background

During their meeting in Washington, D.C. in January 1965, the Prime Minister of Japan and the President of the United States gave purposeful recognition to their common and urgent concern for the health and well being of all the people of Asia. A new bilateral program was proposed and named the United States-Japan Cooperative Medical Science Program (U.S.-Japan CMSP). The two heads of state agreed to undertake a greatly expanded, joint cooperative research effort in the biomedical sciences, concentrating on health problems of recognized importance in Asia.

Initially, both the United States and Japan appointed prominent medical advisors from their respective countries as Delegates to a joint U.S.-Japan Cooperative Medical Science Program Committee. These consultants continue to review jointly the Program's objectives, operations, and accomplishments. This Joint Committee has met annually since 1965 alternating between locations in the United States and Japan. The most recent meeting was held in Tokyo on September 3-4, 1970.

The Program is concerned with the diseases of substantial health importance in Asian countries. The diseases or categories of diseases are: 1. cholera, 2. leprosy, 3. malnutrition, 4. parasitic diseases (schistosomiasis and filariasis), 5. tuberculosis, and 6. viral diseases (rabies, dengue-hemorrhagic fever, and certain arbovirus diseases).

The overall U.S.-Japan Cooperative Medical Science Program operates within a bilateral government framework. Nevertheless, it may involve scientists and facilities in third countries, and/or collaboration with international or other organizations. Joint third country activities are approved in advance by the U.S. and Japanese Delegations.

The relevant regions in Asia are not specifically defined, but are generally understood to include the Republic of Korea on the north, India and Pakistan to the west, and other adjacent nations in the broad Pacific basin.

The United States Delegation to this Committee is appointed by our Secretary of State, and Japan's Delegation by the Japanese Ministry of Foreign Affairs. The Delegations advise their respective governments on the scope, direction and other broad aspects of the Program, and develop review procedures to assure fulfillment of the purposes for which the Program was established. Jointly the United States and Japanese Delegations have designated the six areas of research which fall within the scope of the overall Program.

In Japan the Program activities are the responsibility of the Ministry of Health and Welfare and the Ministry of Education. In the United States the operational aspects of the Program have been delegated to the National Institutes of Health (NIH), Department of Health Education, and Welfare. The United States and Japan maintain separate secretariats.

For each of the six areas selected for study, ten-member joint Panels, consisting of five scientists from each nation, were established to delineate more specifically areas for mutual cooperation and to carry on Program activities. Briefly summarized, these activities may be grouped into the following three categories: support of research, joint scientific conferences, and exchange of persons. In addition, each Panel prepares an annual report of progress for review by the Joint American and Japanese Delegations at their annual, joint meetings. The Panel scientific meetings, as do the Delegation Meetings, alternate annually between locations in the United States and Japan.

The United States and Japan each support the cost of their own scientific projects and meetings. Research by U.S. scientists is funded by the United States and that of Japanese scientists by Japan. Exchange of scientists between the two countries is encouraged as a matter of principle and is supported by official or unofficial agencies of either country. The U.S. does not fund research in Japan nor grant fellowships to Japanese scientists under this Program, nor does Japan fund U.S. research or fellowships for Americans as a component of their Program-sponsored activities.

The National Institutes of Health, based upon a delegation of Presidential authority under Public Law 86-610 and on the availability of funds, provides:

- support of scientific projects
- organization, funding, and conduct of scientific meetings held in the United States
- financial support to the United States Panel members and consultants attending meetings related to the Program
- staff and operating costs of the U.S. Secretariat

The Japanese Ministries of Health and Welfare and of Education assume a similar responsibility for their counterpart activities, i.e. research projects, meetings held in Japan, Japanese Panel members and consultants, and for the Japanese secretariat.

Highlights of the Past Year

CHOLERA

During the past year, significant progress has been made by the U.S. Cholera Panel in collaboration with the NIH Cholera Advisory Committee in each of three major priority areas, including the development of a more effective vaccine, the delineation of the mechanism by which the cholera toxin causes intestinal fluid loss, and the isolation of bacterial exotoxins responsible for non-vibrio, cholera-like disease.

In regard to vaccine development, additional laboratory studies have confirmed that antitoxic immunity, induced by inoculation of somatic-antigen-free toxin, confers a high degree of protection against challenge with massive doses of virulent Vibrio cholerae organisms. As the result of rapid improvement in techniques for the production and purification of cholera exotoxin, large-scale production of a cholera toxoid, free of somatic antigen, is underway. Preliminary testing of a pilot lot of toxoid uncovered the spontaneous dissociation at room temperature of exotoxin from the toxoid. Therefore, further investigations designed to stabilize the purified toxoid are in progress. Future field trials are planned to assess the efficacy of this cholera toxoid in a cholera endemic area.

Rapid strides have been made toward the precise delineation of the mechanism by which the cholera exotoxin causes secretion of fluid and electrolyte by the gut. Following the initial suggestion that the exotoxin induced electrolyte secretion is mediated by cyclic adenosin monophosphate (AMP), it has been clearly demonstrated that gut electrolyte secretion can be initiated by other agents which increase intracellular cyclic AMP levels, and conversely, such intestinal secretion can be inhibited by at least one agent which decreases intracellular cyclic AMP. As the result of studies in this area, number of agents known to inhibit intracellular cyclic AMP accumulation are now being investigated to ascertain their possible practical value in rapidly blocking the gut fluid loss in the cholera patient.

Except during major cholera epidemics, the so called cholera-like diseases result in greater morbidity and mortality throughout South and Southeast Asia than do endemic cholera infections. The pathogenesis of this group of similar diseases is under intensive investigation. Exotoxin produced by cultures of E. coli bacteria isolated from patients with cholera-like illness cause effects qualitatively similar to those of the cholera exotoxin. Also, studies have shown that the ability to produce this exotoxin may be transmitted, apparently by plasmid transfer, from toxigenic to non-toxigenic E. coli strains. A number of carefully designed studies of the pathogenetic and immunologic aspects of cholera-like diseases is currently underway. If it can be shown that a single bacterial exotoxin, or a family of closely related exotoxins, are responsible for most of the cholera-like illnesses, there is then, as in cholera, a very real possibility of developing a toxoid which may confer protection against this devastating group of cholera-like diarrheal diseases.

LEPROSY

The U.S. Leprosy Panel continues to make progress in major priority areas including pharmacology of antileprotic drugs, chemotherapy trials with promising new compounds, cultivation of the leprosy bacillus *in vitro* and *in vivo*, and the role of the immune mechanism in this disease.

The pharmacology of dapsone (DDS or 4,4'-diaminodiphenylsulfone) was the first to be studied in depth because this drug has been used world-wide for many years in the treatment of leprosy. Recent investigations have shown that the principal metabolite of DDS in plasma, MADDS (monacetyl DDS), is enzymatically acetylated by the same enzyme system which acetylates isoniazid and sulfonamides. The speed with which a patient acetylates, slow versus fast acetylator, and the therapeutic response of each acetylator type to DDS therapy, is not known. Studies are in progress in leprosy endemic areas to determine the distribution of acetylator phenotypes. Experimental data already reported from Cebu, Philippines show similarities to patients in the United States regarding plasma levels of DDS, ratios of MADDS, to DDS, and distribution of rapid and slow acetylator phenotypes.

Preliminary information obtained from a clinical trial using various dosages of clofazimine (B663) indicate that this drug is effective, although long-term follow-up will be necessary before a final evaluation can be made. Unfortunately, B663 produces a deep reddish pigmentation in the skin. Therefore, it is unlikely that it will be used extensively, although in cases resistant to DDS therapy, B663 could be of value. The resistance of *M. leprae* bacteria to DDS may be a problem, and a number of drug resistant cases have been reported. Therefore, in an attempt to prevent the emergence of drug resistance, combinations of antileprotic drugs will be used in chemotherapy trials. Specifically, a trial is planned using DADDS and rifampin, a drug used successfully in treating tuberculosis. Other promising antileprotic compounds screened by the mouse foot pad technique will be included in future trials. One contractor is synthesizing derivatives of DDS in an attempt to increase the effectiveness of the parent compound.

To date, *in vitro* cultivation studies of the leprosy bacillus have not been rewarding. A number of tissue culture systems using different media, supplemented with various chemical and biological additives, have been tried without success. However, these studies will be continued using new approaches and employing organisms obtained from human biopsies. The *in vivo* cultivation of the causative organism in the mouse foot pad continues in use for laboratory studies. Thymectomized irradiated or thymectomized ALS treated mice are not being utilized in an attempt to increase bacterial yields and to produce disease similar to that found in human leprosy cases.

The immunology of leprosy is particularly interesting, since leprosy patients often demonstrate deficient cellular immunity. This deficiency is characterized by decreased reactivity to tuberculin injected into the skin and by decreased ability to develop new, delayed-type sensitivities. Foreign skin graft rejection is also delayed. It would appear that the thymus-dependent lymphocytes of the lepromatous patient are deficient. Attempts now focus on the mechanisms underlying this deficiency.

To encourage and facilitate leprosy research, a number of research materials, otherwise difficult to obtain, are provided upon request to qualified investigators. Contractual arrangements have been made to obtain biopsy specimens from endemic leprosy areas. In the future, thymectomized irradiated and thymectomized ALS treated mice will also be available to scientists interested in studying this disease which still remains as an important threat to human health throughout Asia.

MALNUTRITION

The third joint meeting of the United States and Japanese Malnutrition Panels was held in Tokyo, Japan, November 5, 1970. Progress in each priority area was reviewed. There followed an International Symposium (November 6-7) on "Criteria and Methodology for Assessment of Nutritional Status" which focussed upon most recent advances and future needs for proper evaluation of the nutritional status of man. Joint Panel discussions were directed largely to the problem of how present and anticipated new knowledge in nutrition can most effectively find application in, and propitiously direct, national policies pertaining to improvements in nutrition and health in developing countries. Because of its world-wide importance, this topic was proposed for critical discussion as an International Symposium to be coordinated with the Joint Panel meeting in Cambridge, Massachusetts in September of 1971.

Some of the major directions taken, and progress made, in the program of the U.S. Panels are summarized as follows:

- Malnutrition and Mental Development
  Several research projects supported in a number of countries have substantially extended earlier evidence that severe malnutrition (Marasmus, Kwashiorkor) during the first two or three years of life, even though dietary adequacy is later provided, has a lasting detrimental effect upon intelligence and behavior in later life. It is anticipated that highly sophisticated neuropsychiatric and neurophysiologic methodology currently being developed and applied will provide the basis for more exacting quantification of brain functions and allow more precise evaluation of the influence of early malnutrition thereon.

- Malnutrition and Infection
  There has been a dramatic fall in morbidity rates from infectious diseases observed in villages in Northern India receiving both nutritional supplements and medical care. This was not seen when medical care alone was provided. Better control of infections which so commonly thwart recovery from severe malnutrition in infancy is under careful study.

- Nutritional Anemias
  Research in Thailand and Malaysia has demonstrated a high prevalence and frequency of iron and protein deficiency in children, and of folic acid deficiency in pregnant women.

Other research in progress endeavors to explain, and find means for correcting, the poor absorption of iron from cereal grains.

- Protein Sources and New Protein Foods
  There has been continued, careful evaluation of a variety of new low-cost food formulations for meeting needs of certain population groups. This research complements the ongoing efforts of WHO, FAO, and UNICEF.

- Mechanisms of Adaptation to Nutrition
  There is new and important evidence from Taiwan that Oriental populations can adapt more effectively than can Caucasian populations to significantly lower amounts of dietary protein per kilogram of body weight.

- Natural Toxins in Foods
  In Thailand, Malaysia and Hong Kong investigators have found an unexpectedly high frequency of contamination of common foods with toxins of Aspergillus molds. A possible relationship of these mold toxins to liver carcinoma and to Reyes syndrome, a fatal disease of children, is under intensive study.

- Genetic Improvement in Cereal Grains
  Important advances have been made in the production of varieties of rice, corn, wheat, and sorghum with increased protein and improved amino acid content. Primarily, this research methodology has involved genetic processes, but also chemical treatment of the growing plant has been tried with success.

PARASITIC DISEASES

Research under the Parasitic Diseases Panel is confined to two parasitic infections of very great importance in Asian countries: schistosomiasis and filariasis:

Schistosomiasis

During the past year, new compounds have been developed and tested against Schistosomiasis. One study is elucidating the relationship between chemical structure and configuration compared against antischistosomal activity *in vitro* of heterocyclic compounds. One compound which had previously been an effective anti-schistosomal agent elicited drug resistence. The mechanism of this resistance is currently being studied since this phenomenon has never been observed with other anti schistosomal compounds. Investigations continue to be focused on elucidating various biochemical metabolic pathways of schistosomal larval forms. To date, this research has demonstrated closely similar features of analogous pathways of carbohydrate and nucleic acid metabolism among the miracidia, cercariae and adults of *Schistosoma mansoni*.

In the area of pathology studies, the fine structure of the schistosome has been delineated. One investigator has placed emphasis in the past year on the biochemistry and antigenic composition of the schistosome egg, separating fractions and determining their role in the pathology of the disease. In a study testing the hypothesis that the schistosome egg is primarily responsible for the occurrence of overt disease, another investigator showed that a drug, which suppresses egg production by schistosomes, not only prevents the development of hepatosplenic schistosomiasis in heavily infected mice, but markedly ameliorates early disease.

With respect to the control of schistosomiasis, a field study is being carried out on the feasibility of biological control which would take the form of blanketing the snail population with mass-produced eggs of a dominant parasite (not pathogenic to man) which could effectively eliminate the human schistosomes from the snail vectors. In addition, a contract was awarded to further the development of an economic "bait" formulation containing molluscicidal compounds for the snail vectors of schistosomiasis in order to eliminate the environmental contamination resulting from current snail control procedures.

A contract for the supply of schistosome material and snail vectors has continued to play an active and important role in support of this research.

Filariasis

Efforts have continued to synthesize antifilarial drugs, and these compounds have been thoroughly tested in animal models. Although no compounds have been demonstrated to be more active than diethylcarbamazine, these studies are adding to the limited knowledge in this field. In June 1970 a workshop was held on the chemotherapy of filariasis. At this workshop, parasitologists, organic chemists, clinicians immunologists, biochemists, pathologists and pharmacologists discussed current knowledge and made recommendations for future studies. One recommendation which is presently being implemented via contract support is the synthesis of isotopically labelled diethylcarbamazine for supply to investigators interested in determining the mechanism of action of this widely used antifilarial compound. A more rational approach to filariasis chemotherapy is contingent upon the delineation of this mechanism of action.

Experimental infection of small animals (gerbils) with Brugia pahangi and the human filarial parasites Brugia malayi and Wuchereria bancrofti have made possible studies hitherto either impossible or prohibitively expensive. The production of experimental elephantiasis in dogs has led to a clearer understanding of the pathogenesis of this misunderstood filariasis syndrome. Another investigator has recently initiated a study on the changes which might occur during the course of development and maturation of the filarial parasites. This study involves measurements of blood pressure, electrocardiography, blood gases, and respiratory analyses.

A contract to supply filarial materials has stimulated fifteen new investigators to begin work in this field. Another contract is directed towards the control of vectors of filariasis by supplying investigators with various genetically marked strains of mosquitoes which serve as vectors carrying filariasis. These strains are of increasing significance as further limitations are set on the use of pesticides.

TUBERCULOSIS

The U.S. Tuberculosis Panel has purposely limited its workscope in order to concentrate on the pathogenic and immunologic aspects of tuberculosis.

The development of vaccines to prevent tuberculosis is hampered by the lack of a reliable animal model test system. Although many test systems are in use, they give conflicting results. Therefore, an important objective of the Program is to find a practical animal model which gives results relevant to the immunity that exists in humans against naturally acquired tuberculosis infection. With such a model, improved tuberculosis vaccines, both living and non-living, could be developed for clinical use. Currently, two contractors using different animal systems are working toward resolving this important, but difficult problem.

The understanding of immunity and delayed hypersensitivity at the cellular level is another area of concern. The cells involved, changes within those cells, the sequence of certain critical events, the chemical mediators, and the constituents of the tubercle bacilli responsible for initiating all of these events are of great interest. Data reported indicate that the ingestion of living mycobacteria or cell wall fractions by the macrophage cells provokes into action the small, short-lived lymphocyte by a pathway not yet defined. The small lymphocyte develops a number of factors capable of mobilizing macrophages and inhibiting their migration. The macrophage-inhibiting factor has been isolated and chemical characterization studies are in progress. Other chemical mediators in the chain of events have been isolated, but are not as well characterized. It is apparent that it is the alteration in the small lymphocyte and not the macrophage which produces a state of delayed hypersensitivity and probably immunity. However, the relationship of the two phenomena is still the subject of much debate in the scientific community.

In order to encourage and stimulate studies of the immunology of tuberculosis and the basic mechanisms of cellular and humoral responses to infection and disease, special reagents, difficult to prepare and not readily available, have been produced under contract for the U.S.-Japan Program to be distributed to qualified investigators. These include: living, frozen mycobacterial cells of the Glaxo vaccine strain of BCG; lyophilized PPD tuberculin; a large number of fully characterized mycobacterial strains; and specific rabbit antisera against many strains of mycobacteria. The latter sera are most helpful in identifying and differentiating various strains of mycobacterium from unclassified (atypical) mycobacteria.

VIRAL DISEASES

During the past year, the U.S. Panel on Viral Diseases decided that in order to utilize available funds more effectively, the scope of the Panel's interests should be narrowed. As a result, the guidelines related to program relevance were altered to include only studies on rabies, and on arboviruses, with emphasis on dengue and the pathogenesis of hemorrhagic fever.

Rabies

A rapid and easily performed passive hemagglutination procedure has been developed for the measurement of rabies antibodies. Tissue culture grown virus is concentrated and partially purified by zinc acetate and coupled to goose erythrocytes with chromium chloride. Serial dilutions of heat inactivated adsorbed sera are added to the sensitized erythrocytes and held at 4° Centigrade for one hour before examination for hemagglutination. A correlation coefficient of 0.81 was found between the results of this and mouse neutralization tests. An explanation for the occasional failure to isolate virus in mice using material positive by immunofluorescence is that specific IgG is present in the inoculated material and neutralizes the virus in the homogenate. The source of the globulin is believed to have been serum. The development of extra-neural salivary gland street viruses in both spotted and striped skunks has been followed in the same species by histopathology, fluorescent antibody, and electron microscopy procedures. To date, virus has been detected only in salivary glands, brain, pancreas, and adrenals. In addition, contract support has been given to a study of the feasibility of utilizing steric exclusion chromatography in the purification of rabies virus vaccines.

A Joint Conference on Rabies, held in October 1970 in Tokyo, dealt with the nature of the rabies virus, the pathogenesis and diagnosis of rabies, epidemiology and control measures as well as some of the pitfalls in diagnosis. As a result of this conference, the U.S. Panel is embarking upon a concentrated contract program centered around the development of an improved post-exposure rabies vaccine.

Dengue

Three structural proteins of dengue-2 virus have been identified and assigned molecular weights.

Monkeys have been subclinically infected with various dengue virus strains. The biological behavior patterns of unadapted and mouse-adapted strains of virus in monkeys are different for different types of dengue viruses. Mice infected with unadapted dengue strains of the same type in some instances did not appear to be antibody dependent.

A pig kidney tissue culture system has been developed for study of all mouse-adapted dengue types. A reproducible plaque assay system and a plaque neutralization test have been standardized and are in routine use in one laboratory.

A small workshop on the pathogenesis of dengue hemorrhagic fever was held in the United States in November 1970 to which scientists and clinicians from several related disciplines were invited to discuss hypotheses and to suggest ideas for further study of dengue and dengue hemorrhagic fever.

Arbovirus

Serological studies on the grouping of arboviruses has continued. Preliminary grouping of tick-borne viruses reflects satisfactory progress. New viruses have been examined from the group of Phlebotomis fever viruses, the VSV-rhabdovirus group, the Simbu group, the Anopheles A group of South America, etc. The very hazardous new agent. Lassa Fever Virus, has been shown to be related morphologically to the Tacaribe group of viruses. Mosquito cell lines may prove useful for distinguishing mosquito-borne arboviruses from tick-borne arboviruses.

Peptide mapping of purified concentrates of variants of Sindbis virus failed to demonstrate significant differences. Amino acid analysis of purified envelope and nucleocapsid components will therefore be necessary as a further step in attempting to identify a chemical basis for Sindbis virus variation.

Genetic studies of mosquito vectors of arboviruses continue to yield intriguing information. Cytoplasmic incompatibility is given as the basis for elimination of a population of *Culex fatigans* in a field trial conducted in a village in Burma. The results of studies on selecting a strain of *Culex tritaeniorhynchus* resistant to transmission of Japanese encephalitis virus are encouraging. Ninety percent of the females in selected lines were found refractory to infection.

Research Conferences and Workshops:

In addition to Program elements involving research grants and contracts, the U.S.-Japan Cooperative Medical Science Program includes a provision for regular scientific conferences and workshops. These include joint meetings with Japanese counterpart scientists as well as special conferences for American investigators working in the scientific areas of specific interest to a given Panel. These workshops and conferences serve to stimulate and plan new scientific approaches to the six disease categories within the Program, and they also facilitate the timely exchange of research results, methods, and protocols. The Joint U.S. and Japanese Panels meet annually, alternating between locations in the United States and Japan. A notation of conferences and workshops sponsored during FY 1971 follows:

Meeting Title and Location	Sponsoring Panel
August 31 & September 1-2, 1970 Joint Cholera Conference Fuji National Park & Hakone, Japan	Cholera
August 31 & September 1-2, 1970 Joint Tuberculosis Conference, Tokyo, Japan	Tuberculosis

Meeting Title and Location	Sponsoring Panel
October 12-15, 1970 Joint Conference on Rabies Tokyo, Japan	Viral Diseases
November 4-5, 1970 Workshop on the "Pathogenesis of Dengue Hemmorrhagic Fever" San Francisco, California	Viral Diseases
November 5-7, 1970 Joint Malnutrition Conference Tokyo, Japan	Malnutrition
November 7-11, 1970 Joint Parasitic Diseases Conference Tokyo, Japan	Parasitic Diseases
November 10-12, 1970 Joint Leprosy Conference Tokyo, Japan	Leprosy
January 12-13, 1971 Malnutrition Planning Session Washington, D.C.	Malnutrition
March 10-12, 1971 Sixth Annual Leprosy Research Conference Atlanta, Georgia	Leprosy
April 26-27, 1971 Workshop on "Intramolluscan Development of Larval Schistosomes" Boston, Mass.	Parasitic Diseases

II. The International Centers for Medical Research and Training (ICMRT) Program

Program Description:

In response to the expressed desire of the Congress (P.L. 86-610), a program for the International Centers for Medical Research and Training was established in 1960 by the National Institutes of Health "to advance the status of the health sciences in the United States and thereby the health of the American people" by expanding collaborative research and research training between U.S. universities and selected foreign institutions and investigators.

The four grants funded under this Program are awarded for five years with continued support contingent upon program review at the end of each year of operation. One of the most important features of this program is that of long-term support, as contrasted with the transiency which has characterized a number of other international projects. This is a necessary ingredient for the full development of university careers in science which will effectively utilize the international medical experience of individual scientists. A major advantage of this program is that it provides opportunities for established and highly competent investigators to exploit valuable research opportunities abroad without losing their university affiliations and faculty appointments. The long-term support of the ICMRT's should not be construed as foreign aid since the primary objective is a collaborative effort in biomedical research and research training of relevance to the health of the American people and to the U.S. scientific community. The four current ICMRT grants were renewed for an additional five years beginning in FY 1970, and at the same time the NIH phased out ICMRT grant support of the LSU Center in San Jose, Costa Rica.

Purpose and Scope:

The ICMRT program has as its principal objective the provision of high quality research training and career motivation of Americans in the Broad fields of geographic medicine, and in response to the special opportunities existing within each ICMRT establishment. The achievement of the research training goal for each ICMRT unit depends largely on the actual conduct of research by established investigators who can serve as mentors for those less experienced scientists participating primarily in a learning kind of international research experience. The length of the training period is a descretionary concern of the ICMRT program director, and is obviously determined by both the interests of the prospective scientist-trainee and the opportunities available within the given ICMRT unit. Always the emphasis is upon the quality rather than the quantity or duration of the research training experiences.

The aggregate ICMRT units are serving increasingly as a national resource for utilization by both trainee and trained scientists to increase the pool of investigators with a sustained career interest in international or geographic biomedical research. In this regard, the ICMRT program directors are encouraged to establish a selective, multidisciplinary scientific program. Their activities accommodate a number of parent university departments other than the department which primarily sponsors the ICMRT operation. Particular advantage is taken of special strengths within the overall parent university, including opportunities in fields outside of the classical confines of tropical medicine.

During the current year the NIH has initiated a new program element within the overall ICMRT framework. Designated as the ICMRT Research Associate Program (IRAP), this centrally directed activity is specially designed to recruit both experienced investigators and prospective trainees on a national basis and specifically from universities other than the ICMRT sponsoring institution. The administrative structure of an ICMRT remains relatively flexible and is designed, constructed, and modified to complement in an optimum manner the sponsor's resources and the overseas opportunities and environment.

As far as is possible, the ICMRT core grant is used for training and research support at the off-shore ICMRT site rather than at the parent, domestic university. In this regard it is accepted that some training preliminary to an overseas assignment is frequently necessary or desirable, and as many items of equipment and supplies as feasible are purchased in the United States.

Program:

The five institutions participating in the ICMRT program at present, and their respective areas of research and training interests are as follows:

Johns Hopkins University (Baltimore, Maryland)

Program Director: Dr. Frederik B. Bang

Johns Hopkins University with the participation of the School of Hygiene and Public Health and the Department of Medicine is collaborating with the Calcutta School of Tropical Medicine, the All-India Institute of Hygiene and Public Health, and the Postgraduate Institute for Medical Education and Research in Calcutta, India. Program emphasis is directed toward the epidemiology and management of human diseases of large populations and the ecology and behavior of animals which live in comparatively close association with man.

Studies are in progress on the year-round distribution of respiratory and enteroviruses in normal children in rural communities. Other research includes the factors influencing distribution of hookworm, the development of antigens for skin tests in leprosy, and the ecology of the economically important bandicoot rat (B. bengalensis).

Intensive biochemical research in cholera is also in progress. This work includes antibiotic and oral therapy, cholera in children, comparison of classical and El Tor cholera, and a number of very significant experimental pathophysiological studies.

Louisiana State University (New Orleans, Louisiana)

Program Director: Dr. J. Clyde Swartzwelder

Louisiana State University with the participation of the School of Medicine is collaborating with the University of Costa Rica in San Jose. The location of this research center provides an excellent opportunity for a statistical survey and extensive pathological study of a number of diseases prevalent in San Jose and other parts of Costa Rica.

Epidemiologic studies of diarrheal diseases among 300 families in Costa Rica are in process. A strong relationship has been found between the diarrheal diseases and coxsackie B virus. Another study concerns the chemotherapy of hookworm wherein the prevalence of hookworm dropped markedly when individuals were treated with combinations tetrachloroethylene and piperazine.

Other studies have included investigations of a large outbreak of infectious hepatitis, investigation of the effect of passive immunizations with gamma globulin upon the rates of diarrhea illness and death among infants, and an evaluation of Thiabendazole as an antihelmenthic. The epidemiology of a new paragonimus species has recently been delineated.

Following review for a third 5-year period of support, the NIAID has decided to phase out ICMRT grant support of this unit. No FY 1971 funds have been provided, although the grant has been extended without funds to help ease the impact of decreased NIH support.

University of California (San Francisco, California)

Program Director: Dr. J. Ralph Audy

The University of California with the participation of the Hooper Foundation, the School of Public Health, and the Medical School, is collaborating with the Institute for Medical Research, Kuala Lumpur, Malaysia, and the University of Malaya. This International Center has placed strong emphasis on infectious diseases transmitted from animals to man. Because of the long standing research interest and experience in studying the fundamental problems of human ecology at the University of California, a collaborative program has been developed embracing medical zoology, microbiology and parasitology. The scope of this collaborative endeavor has now been expanded to include related socio-cultural research and medical genetics.

Long-term research projects in Malaysia and California are based on three general laboratory programs: the Arbovirus Research Laboratory, the Parasitology Laboratory, and the Medical Anthropology and Rural Health Research project.

The arbovirus research is focused on the epidemiology and ecology of dengue virus and the relationship of this virus to mosquito-borne Hemorrhagic Fever. The parasitological research is based on studies of trematode ecology and interactions of various parasites in the snail host and the ecology of Malayan pentastomes or tongue worms. The snail trematode antagonism studies are directed toward the ultimate biological control of schistosomiasis, and this ICMRT represents a leading world resource for this important field research. Investigators from this ICMRT have also studied the histopathology of host reaction to acarine parasites, the relationship of monkeys to human malarial infection, and jungle cycles of scrub typhus, Q fever, and tick typhus.

Abundance, distribution, life cycles and ectoparasites of rodents closely associated with man have been studied. Work on the possible role of the Indian fruit bat as a reservoir of human viral infections has been completed. Other field studies include the investigation of bacteriae and viruses pathogenic for man in monkey populations.

University of Maryland (Baltimore, Maryland)

Program Director: Dr. David F. Clyde

The University of Maryland with the participation of its School of Medicine, and collaborating with the Institute of Hygiene, Lahore has undertaken a varied research program in West Pakistan. Comprehensive studies of arthropod-borne diseases have been set up, as well as research in the rickettsial diseases which are endemic in this area. Since recent evidence indicates that mites, present on wild rodents, are important agents in the transmission of typhus, geographic and seasonal distribution of scrub typhus is being investigated.

More recent research includes studies of mosquito-borne viruses in man, the role of mosquitoes and ticks as vectors in human disease, identification of vertebrate hosts in the transmission of scrub typhus, and evaluative study of malaria transmission and new control modes, and a comprehensive epidemiologic survey of smallpox. In addition, a detailed investigation of several different clinical entities, e.g. bleeding disorders in humans, is now under way, as well as investigations in basic immunologic aspects of the bacterial infection and disease. New projects, with some expansion into East Pakistan, include filariasis and tuberculosis research.

Of great value have been mosquito genetic investigations of a comparatively unstudied vector of Japanese Encephalitis Virus. Knowledge about the genetics of this mosquito, Culex tritaeniorhnychus, now approaches the abundance and high quality of information previously known about two other mosquito vectors of human disease. This new information has been sought to establish a scientific basis for the ultimate biologic control of human Japanese encephalitis.

Because of uncertainties about the long term ability of the University of Maryland to continue its operation in Pakistan, an alternative ICMRT site is currently being sought. A new location in Latin America seems likely.

Tulane University (New Orleans, Louisiana)

Program Director: Dr. Paul C. Beaver, Acting

Tulane University, with the participation of the Departments of Tropical Medicine and Public Health, other departments of the Medical School, and the Department of Anthropology and Sociology, is collaborating with the Universidad del Valle, Cali, Colombia. The research work is in two general areas: causes, prevalence and distribution of human diseases and animal diseases with human relevance, and physical environments which determine ill or well-being.

Studies near completion include a longitudinal survey of diarrheal diseases of infants and young children. Although nutritional, environmental and chemical factors may be associated with this symptom complex, studies are now under way to discover the extent to which bacteria, viruses or parasites are involved. Another investigation in experimental epidemiology relates to the frequent occurrence in Colombia of tetanus in newborn children and its effective prevention through the use of tetanus toxoid administered to women during pregnancy. Parasitic diseases research has centered principally around the following diseases: trypanosomiasis, malaria, filariasis, and amebiasis. The local epidemiology of Venzuelan Equine Encephalitis has been studied and documented following a recent epidemic. The Virus Group also has a continuing interest in rabies. A comprehensive evaluation of bacterial meningitis treatment regimens is in process. Finally, a multidisciplinary study of frustrated suicides has recently been initiated. New projects are concerned with maternal and child health, family planning, and include health services research. Socio-cultural studies concerned with Malayan attitudes toward health and nutrition and the influence of native and western practitioners of medicine upon these attitudes have been undertaken by a number of variously trained, medically oriented social scientists.

2. CONTRACT NARRATIVES

U.S.-Japan Program

JEFFERSON MEDICAL COLLEGE (NIH 70-2188) .... Cholera

Title: Vibrio Reference Laboratory

Project Director: Dr. Harry L. Smith, Jr.

Project Officer: Dr. Earl S. Beck

Objectives: The objectives of this contract are to 1) maintain a reference collection of *Vibrio cholerae* and other vibrios and to supply strains upon request; 2) receive and identify serologically new vibrio isolates; and 3) prepare and distribute to qualified investigators cholera typing antisera.

Major Findings: The contractor maintains a reference library containing some 5,000 cultures of *Vibrio cholerae* and other vibrios. Approximately 200 isolates were received for typing and characterization. In addition, 669 vials of lyophilized cholera diagnostic sera and 11 cultures from the reference library were sent to investigators in the United States and in other parts of the world. To reduce serum wastage in the field, the investigator developed a method for lyophilizing serum on paper discs. Also, preliminary testing in sheep indicate that this animal may be useful in preparing large quantities of cholera diagnostic sera.

Significance to NIAID Program and Biomedical Research: It is important in cholera research to have a laboratory which possesses the competence and the expertise to identify accurately and to differentiate *V. cholerae* from other enteric organisms. The need for reference cultures and for typing sera is important and essential to an active research program. Many investigators involved in the 7th cholera pandemic have used and depend upon the services and reagents of this laboratory.

Proposed Course of Project: Review and evaluation at the end of the contract year for continued support.

Date Contract Initiated: March 1970

Current Annual Level: $18,000

THE JOHNS HOPKINS UNIVERSITY (NIH 70-2051) .... Cholera

Title: Assays of Cholera Toxins and Toxoids in (A) Dogs and Rabbits and (B) Other Assay Systems.

Project Director: Dr. Charles C. J. Carpenter, Jr.

Project Officer: Dr. Robert S. Northrup

Objectives: The contractor assays in several test systems cholera products generated by other contractors who have neither the experience, the expertise nor the facilities to perform these tests effectively. Also, work is continuing on the development of new and improved assay techniques. The contractor is an important component in a well-defined collaborative program.

Major Findings: During the first contract year, the principal investigator and his associates assayed samples of purified cholera toxin, toxoid and sera for other investigators. Assays were carried out in dogs, rabbits and in isolated, viable, rat epididymal fat cells. The latter test, employed specifically to measure cholera toxin and serum ENA, was developed under the contract and is being comparatively evaluated in the hope that it will replace the expensive and time consuming animal model. The effect of parenterally administered toxin and toxoid to dogs followed by a live oral challenge was studied. Although the preparations in the proper concentrations protect, the duration of the immune response is still to be determined. Parenteral administration of toxin in a dog was found to cause hyperglycemia, increased serum alkaline phophatase levels, leucocytosis, hyponatremia, weight loss, enhanced lipolysis of fat cells, altered SGOT levels and even death. All toxoid preparations prior to human use will be tested in a similar manner for evidence of toxin.

Significance to NIAID Program and Biomedical Research: This facility will serve as a central testing laboratory for other investigators involved in cholera research. The service will provide uniform and rapid testing of submitted samples, thereby facilitating and expecting research progress.

Proposed Course of Project: Review and evaluation at the end of the contract year for continued support.

Date Contract Initiated: December 1969

Current Annual Level: $93,000

THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK (NIH 70-2091) ....
Cholera

Title: Establishment of a Laboratory for the Evaluation Measurement of Cholera Toxins, Antitoxins and Toxoids.

Project Director: Dr. John Craig

Project Officer: Dr. Robert Northrup

Objectives: Establishment of a laboratory to perform PF (permeability factor) titrations of cholera toxins, antitoxins and antitoxin combining power of cholera toxoids, toxins and sera. Also, to assay the immunogenicity of cholera toxoids by the mouse protection test, and the residual toxicity by mouse inoculation.

Major Findings: During the past year, the contractor performed approximately 120 PF titrations per month. Because of the instability of the present lots of toxoid and the need to produce additional lots using a variety of methods for purification and detoxification, the number of samples to be tested will be doubled. In addition, the contractor is studying the mouse as a possible model for investigating the mode of action of cholera toxin. Mortality dose-response curves have been determined by administering crude and purified toxin intravenously to 21-25 gram femal mice. Strong preliminary evidence indicates that the PF and the lethal factor are identical.

Significance to NIAID Program and Biomedical Research: The contractor will fulfill program need for comparative testing of materials under standard conditions for other research laboratories involved in cholera work.

Proposed Course of Project: Review and evaluation at contract termination for continued support.

Date Contract Initiated: April 1970

Current Annual Level: $118,265

WYETH LABORATORIES, INC. (PH43-68-673) .... Cholera

Title: Isolation, Purification and Characterization of Choleragenic Toxin and Investigation of its Immunologic Properties.

Project Director: Dr. Benjamin A. Rubin

Project Officer: Dr. John C. Feeley, DBS

Objectives: The contractor will determine optimal conditions for:

1. toxin production
2. concentration and purification of toxins
3. detoxifying toxin
4. lyophilizing cholera toxoid

Immunological studies will be limited to the Craig antitoxin units in response to several toxin/toxoid preparations in a single animal species.

Major Findings: A method has been developed for providing highly purified cholera toxoid. The level of purity achieved is far in excess of the minimum standards set forth by the Cholera Advisory Committee, both in terms of protein content and residual somatic antigen. And it appears to be essentially free of other detectable antigenic contaminants. The method lends itself to large scale production procedures and appears to be reliable and reproducible. Overall yields of toxoid antigen of about 50% are feasible and easily attainable.

The procedure essentially requires two basic fractionation techniques. The initial concentration is achieved by fractional precipitation in the presence of metaphosphate. With this procedure, 90% of all Folin-positive contaminants can be removed. The second procedure involves aluminum hydroxide adsorption. The combination of the two reduces the contamination to barely perceptible levels. Further purification may be achieved by high speed centrifugation.

Conversion of both crude and purified toxin to toxoid by formaldehyde was accomplished without loss of antigenicity for laboratory animals. However, in clinical trials the toxoid was found to be unstable, apparently releasing cholera toxin at body temperature. Additional laboratory data confirm the dissociation at room temperature and above. Other methods of purification and inactivation are under study.

Significance to NIAID Program and Biomedical Research: Cholera was one of the six specific disease categories selected for study by the U.S. Delegation to the U.S.-Japan Cooperative Medical Science Program. Results are of direct importance in other NIH-directed activities aimed at development of better immunizing agents against cholera.

Proposed Course of Project: Contract to terminate November 1971.

Date Contract Initiated: February 12, 1968

Current Annual Level: $42,010

WYETH LABORATORIES, INC. (NIH 70-2102) .... Cholera

Title: Production of Cholera Toxoids

Project Director: Dr. Alan Bernstein

Project Officer: Dr. John C. Feeley

Objectives: The contractor will prepare, according to predetermined specifications, a production lot of cholera toxoid free of somatic antigen. The activity of the new product will be compared to that of standard toxin lot 001. Packaging will be done as directed by the Project Officer.

Major Findings: A method has been developed and applied on a production scale for the purification of cholera toxin by a procedure using multiple separation techniques. These consist essentially of a hexametaphosphate (MP) precipitation of toxin from suitably prepared broths, adsorption and elution from aluminum hydroxide with volatile buffers, and lyophilization. The lyophilized material can then be reconstituted in concentrated form and further purified by either ultracentrifugation or ultrafiltration. These techniques have been able to imporve the purity. Subsequently, the toxoiding technique by either formaldehyde or by gluteraldehyde, has been shown to reduce further the immunigenicity of the residual somatic antigen without significantly affecting the immunologic response to the toxoid.

Significance to NIAID Program and Biomedical Research: The product is the result of collaborative research between the Pakistan-SEATO Cholera Research Program and the U.S.-Japan Cholera Research Program. Testing of the toxoid in the field will help establish the present concept that known toxins are responsible for lethal diarrhea and that induced antitoxin can protect man.

Proposed Course of Project: Contract to terminate September 1971.

Date Contract Initiated: March 1970

Current Annual Level: $50,418

GULF SOUTH RESEARCH INSTITUTE (PH-43-68-1272)......Leprosy

Title: Analytical Methods for DDS, DADDS and Their Metabolites

Project Director: Dr. H. P. Burchfield

Project Officer: Mr. George S. Yee

Objectives: The contractor has been conducting a study to develop an analytical method for the determination of nanogram levels of DDS and MADDS and their metabolites in plasma, urine, and tissue from human clinical subjects. The contractor will use gas liquid chromatographic analysis with electron capture detection.

Major Findings: The contractor has developed a method for converting DDS and MADDS to halogen derivatives that can be separated via gas-liquid chromatography and detected by electron capture. The method is sensitive enough for determining nanogram quantities of DDS and MADDS in plasma and urine. In an evaluation study of comparing the GLC method with a chromatographic-fluorometric method, with known samples in a double blind assay, the GLC method was found to be inadequate. DADDS was erroneously found to be present where no DADDS had been included in the sample.

Additional time would be needed to miniaturize the equipment, to adapt the procedure to tissue specimens and to modify the procedure to allow more complete hydrolysis of DDS. Since a chromatographic-fluorometric method was found superior to the GLC method, this contract effort will not be continued.

Significance to NIAID Program and Biomedical Research: The ultimate objectives of this program was to develop methods for determining the metabolic pathways of DDS when administered at very low doses and to establish whether these were the same or different than those followed when DADDS and DDS were administered at high doses.

Proposed Course of Project: This project will be terminated.

Date Contract Initiated: June 19, 1968

Current Annual Level: $60,000

HARVARD COLLEGE (NIH 70-2235) ......Leprosy

Title: Studies of Immunosuppression in Adult-Thymectomized Mice Treated with Antilymphocyte Serum

Project Director: Dr. Anthony Monaco

Project Officer: Mr. George S. Yee

Objectives: The contractor proposes to study and define the course, duration and degree of immunosuppression in adult-thymectomized mice treated with antilymphocyte serum. A schedule for pulsed antilymphocyte serum therapy to maintain prolonged immunosuppression will be determined.

Major Findings: A major portion of the experimental effort expended to date has been to define conditions for the production of a highly potent but non-toxic anti-lymphocyte serum which could be administered at intervals over a long period of time to adult thymectomized mice in an effort to achieve prolonged, perhaps indefinite immune suppression.

To follow this development of the immunosuppressed state in the animal, three techniques were employed: 1) skin allograft survival, 2) challenge with sheep red blood cells, and 3) graft-versus-host reaction. Skin allograft survival was found to be a function of graft placement relative to ALS treatment. Immune suppression as determined by the primary and secondary challenge with SRC paralleled to some degree that defined by skin allograft survial. To maintain suppression of the SRC hemagglutinin response, it was necessary to use a regimen of periodic ALS administration at dilutions which failed to maintain suppression of the allograft response. This finding indicates that to determine optimal regimen for ALS treatment, suppression of a very strong antigenic challenge for a very long period must be utilized as a criterion of effectiveness.

Significance to NIAID Program and Biomedical Research: *Mycobacterium leprae* cannot be grown *in vitro*, and its growth *in vivo* is so slow that experiments must be continued many months before an answer can be obtained. An animal model in which the organism would grow rapidly and produce disease similar to that in humans would do much to speed progress in leprosy research.

Proposed Course of Project: Review and evaluation at the end of the contract year for continued support.

Date Contract Initiated: June 1970

Current Annual Funding: $27,596

LEONARD WOOD MEMORIAL (NIH-69-2003) ....Leprosy

Title: Chemotherapy Trials in Leprosy

Project Director: Dr. Chapman H. Binford

Project Officer: Mr. George S. Yee

Objectives: The Philippine Division of the Leonard Wood Memorial, Cebu City on the grounds of the Eversley Childs Sanitorium, has provided a leprosy research laboratory where clinical trials with drugs of interest can be performed. The Philippine government supports clinical care and management of leprosy patients, whereas studies associated with chemotherapy trials is being supported through research contracts. It is planned that complete bacteriological and pathological backup will be provided b this research facility. Evaluation of several different treatment regimens is presently being provided utilizing the mouse footpad technique, and it is hoped that all animal studies will eventually be conducted at this facility. Because the facility is in a leprosy endemic area, untreated patients are admitted at a rate of approximately 3 per month and are available for chemotherapy trials now being conducted or to be considered at a later date. In addition, through the Philippine Health Department in Manila, skin biopsy material is being provided to U.S. investigators.

Major Findings: The contractor has been able to receive untreated leprosy patients at a rate from 2 to 5 per month which are acceptable for use in a clinical trial. Forty patients have been started on a B663 trial employing various drug dosages at various intervals over a 6 months' period. The biological evaluation, including bacteriological, histopathological and mouse footpad assay are still continuing and should be completed during this contract year. A combined therapy study is being planned with Rifampin and DADDS. The protocol has been reviewed and will be initiated soon. A trial employing Rifampin alone is now in progress.

Significance to NIAID Program and Biomedical Research: This facility, in an endemic leprosy area, provides an important source of patients available to undergo clinical trials with anti-leprotic drugs.

Proposed Course of Project: This project will be funded as long as there is a need for the services provided.

Date Contract Initiated: March 18, 1969

Current Annual Level: $160,000

NATIONAL COMMUNICABLE DISEASE CENTER ....Leprosy

Title: Leprosy Biopsy Collection

Project Director: Dr. Roger A Feldman

Project Officer: Mr. George S. Yee

Objectives: The contractor is to arrange for the collection and distribution of human skin biopsy specimens from leprosy endemic areas. These and other clinical materials will be made available to recommended research laboratories for further studies of interest to the Leprosy research program.

Major Findings: There is a reluctance on the part of clinicians, in leprosy endemic areas, to withhold patient treatment, prior to taking biopsies for shipment. Sources have been requested to insure that each shipment contain a minimum of 3 specimens from different individuals. Therefore, shipments are dependent upon having at least 5 to 10 leprosy patients seeking treatment weekly, with the expectation that a third of these will be untreated cases. Treatment could then be started with a minimum of delay. Most of the specimens received have been from areas with a high incidence rate of untreated patients.

Biopsy specimens have been received from Ethiopia, Thailand, The Congo, Ghana and Korea. Thirteen shipments have been received containing approximately 36 biopsy specimens and these have been sent to six different investigators being supported under grant or contract by the program.

Significance to NIAID Program and Biomedical Research: Through the availability of clinical materials by this mechanism, it is hoped that clinical specimens can be obtained from patients which have not undergone any clinical treatment. Such untreated biopsy specimens are necessary for appropriate baseline studies in leprosy.

Proposed Course of Project: It is planned to continue the service now that sources have been developed.

Date Contract Initiated: February 1968

Current Annual Level: $6,000

Title: Studies on the Pharmacology of Leprosy and the Use of Immunosuppressed Mice in Leprosy Research

Project Director: Dr. Charles C. Shepard

Project Officer: Mr. George S. Yee

Objectives: The contractor will continue to study the activity of DDS and other anti-leprotic drugs using a pharmacokinetic method and employing the mouse footpad technique. Tests for the activity of drugs at different times during the growth period, with known drug dosages, will be used. In this manner drug effectiveness (bactericidal effect) will be determined. The contractor will also continue studies on the use of immunologically suppressed mice. Thymectomized irradiated and ALS treated mice will be investigated.

Major Findings: The contractor has found other drugs besides DDS and B663 to be bactericidal in the mouse footpad test system: Sulfadimethoxine, ethionamide, rifampin, streptovaricin and cephaloridine. Preliminary results indicate that rifampin is also effective in treating leprosy in man. However, the high cost of this drug may preclude its extensive use. Most antimalarial drugs studied were not active as antileprotic agents. Combination drug tests are now in progress utilizing the mouse footpad system, and these data will be particularly useful in studying the apparent emergence of bacterial resistance to DDS.

Significance to NIAID Program and Biomedical Research: By using kinetic methods of analysis, correlated with the mouse footpad technique, the effectiveness of drugs can be investigated in laboratory models before initiating clinical studies.

Proposed Course of Project: This project will be continued to investigate other drugs which appear to be effective against the leprosy bacillus.

Date Contract Initiated: May 15, 1967

Current Annual Level: $70,000

OAKRIDGE NATIONAL LABORATORY .....Leprosy

Title: To supply Thymectomized-irradiated Mice Treated with Bone Marrow to Investigators

Project Director: Dr. Charles C. Congdon

Project Officer: Mr. George S. Yee

Objectives: The contractor provides immunosuppressed adult thymectomized mice to leprosy researchers to investigate the usefulness of this animal model for the growth of M. leprae. In addition, the contractor provides information about immune deficiency in the radiated animal of importance to biology and medical programs in the Atomic Energy Commission.

Major Findings: The contractor has determined that marked defects in the immune response of thymectomized-radiated mice were found. Anatomical studies of lymphatic tissues revealed a gross reduction in size of peripheral lymph nodes at one or two months after thymectomy. The germinal centers were almost completely absent in experimental mice at 1 month but were present in numbers equal to controls at 2 months. Lymphocytes were greatly reduced in the so-called thymus dependant areas of the spleen and lymph nodes at 1 and 2 months following treatment. However some restoration of lymphocytes in the thymus-dependent region of the spleen was noted in the 2 months group. No residual thymus tissue was found in the thymectomized mice at autopsy. Tail skin allografts were rejected more slowly or were not rejected in the experimental mice tested at 1 or 2 months' intervals. Humoral antibody to rat red blood cells was not produced after primary challenge of 1 month.

Significance to NIAID Program and Biomedical Research: Mycobacterium leprae cannot be grown in vitro and its growth in vivo in the mouse footpad model is so slow that many months are needed before evaluation of the assay method can be made. An animal model in which the organism would more rapidly multiply in significant quantities to provide the researcher with live vacilli would do much to speed progress in leprosy research.

Proposed Course of Project: The project will be funded as long as there is a need for the services provided.

Date Contract Initiated: July 1970

Current Annual Level: $47,500

STANFORD RESEARCH INSTITUTE (NIH-70-2286)......Leprosy

Title: Synthesis and Biological Evaluation of DDS Analogs and Sulfonamides

Project Director: Dr. Joseph I. DeGraw

Project Officer: Mr. George S. Yee

Objectives: The contractor plans to synthesize various simple analogs of dapsone, pteridine conjugates of certain sulfones, dapsone analogs modeled after sulfamethoxine and other compounds of close structural relationship to the sulfones to understand better the mechanism of action of DDS and its derivatives. The synthesized compounds will be evaluated *in vivo* and *in vitro*.

Major Findings: Good evidence has been accumulated indicating that sulfonamide and sulfone compounds do act via a related mechanism involving the incorporation of p-aminobenzoic acid (PABA) into dihydrofolate. The addition of either of the latter two compounds reversed the growth inhibitory action of both DDS and sulfamethoxolate.

In addition, the dihydrofolate reductase of the *Mycobacterium sp607* has been partially purified. This enzyme represents the second site of sequential blockade of the *de novo* mycobacterial folate pathway, and its inhibition by a variety of 2, 4-diaminopyrimidines has been studied. The combination of enzymic and whole cell inhibition studies will provide information regarding both inhibitory process and cell penetration ability.

Significance to NIAID Program and Biomedical Research: Dapsone is used world-wide in the treatment of leprosy and is at this time the drug of choice. By synthesizing and studying dapsone analogs and derivatives, an attempt will be made to relate chemical structure with drug activity. This information could ultimately be used to synthesize a more effective drug.

Proposed Course of Project: Review and evaluation at the end of the contract year for continued support.

Date Contract Initiated: June 1970

Current Annual Level: $50,100

STANFORD RESEARCH INSTITUTE (NIH-70-2283)......Leprosy

Title: Metabolism of Dapsone in Leprosy Patients

Project Director: Dr. John H. Peters

Project Officer: Mr. George S. Yee

Objectives: In recent years leprosy researchers have determined that the principal metabolite of DDS in plasma, MADDS, is enzymatically acetylated by the same system which acetylates INH and sulfonamides. The speed at which a patient acetylates, slow acetylator vs. fast acetylator, in terms of therapeutic response of patients to DDS therapy, is not know at this time. To obtain additional information in this physiological response, leprosy patients in the Philippines, Japan, India and other countries will be classified as to acetylator phenotype using sufamethazine and DDS. Also, the effect of continued DDS therapy on metabolism of patients at the USPHS hospital in San Francisco will be investigated. Procedures will be initiated to increase the sensitivity of the fluorometric method to the one nanogram level for tissue specimens.

Major Findings: The contractor has determined that there appears to be a difference in acetylation rates in the Philippine population, and preliminary data indicate rate variation in a Congo population control. Preliminary data indicate that all DDS resistant patients are fast acetylators. This study has been performed on 5 patients and is continuing.

The contractor has established new contacts for phenotyping patients in Japan, India and an American Samoan population. Collaborate efforts will continue utilizing other populations.

In addition to the above studies, the contractor has developed a chromatographic fluorometric method for determining nanogram levels of DDS, MADDS and DADDS in plasma and urine samples. Efforts will be continued to develop this method for tissue specimen and analysis.

Significance to NIAID Program and Biomedical Research: Since DDS is the drug of choice for treating leprosy, it is important to learn as much as possible about the metabolism of this compound within the body. The study should provide useful data for future drug therapy.

Proposed Course of Project: The contract will be reviewed for continued support at the end of the contract year.

Date Contract Initiated: June 1970

Current Annual Level: $55,000

INTERNATIONAL RICE RESEARCH INSTITUTE (PH43-67-726) .... Malnutrition

Title: Improvement of the Protein Content of Rice

Project Director: Dr. B. Juliano

Project Officer: Dr. B. T. Burton (NIAMD)

Objectives: The primary objective of this project is to determine the feasibility of incorporating a high level of protein into commercially produced rice in Southeast Asia. Presently used commercial varieties of rice contain from seven to seven and one-half percent protein. The biological quality of rice protein is fairly high and an increase in the protein level in rice would go far toward alleviating protein undernutrition in countries for whom rice is the mainstay of subsistence. Since 60 percent of the world's population subsists primarily on rice, the implications for an amelioration of the chronic protein shortage in many underdeveloped countries, should this project prove successful, are quite obvious. If the present protein level of rice could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in major areas throughout the world.

Major Findings: Over 100 rice varieties have been identified among the many thousands tested which contain between 13-1/2 to 17-1/2 percent protein. It is now the purpose of the contract to identify, through actual growing experiments under Southeast Asian climatic conditions, the specific rare high protein varieties (from among these 100 varieties) which would also be agriculturally and commercially feasible in a Southeast Asian setting. Thus far six high protein strains of rice (some from Hungary and some from Southeast Asia) have been successfully crossbred with a new rice variety (IR_8) which has been developed previously at the International Rice Research Institute and which possesses near-ideal plant characteristics including disease resistance and a very high yield per acre. Some of the new hybrid lines have shown as high a protein content as the high-protein parent. Others, of fairly acceptable "plant type" have a protein content of +-11 percent, i.e., contain 50 percent more protein than currently produced commercial rice.

Based on the most recent breeding work involving progeny of successful crosses between the six high-protein strains and IR_8 the project staff is encouraged that the protein content of rice is indeed a genetically determined characteristic. (It has always been known that a variety of environmental factors may be important determinants of the final protein concentration in the rice Kernel). At present the experiments are at the F_5 plant stage (which are making F_6 seed kernels). At this point, the plant breeders are suggesting to back-cross the F_6 generation to IR variety parents which produce greater yields per rice paddy area than the current hybrid plants. While the breeders are waiting for the F_5 plants to mature, the biochemical staff is concentrating on elucidating the metabolic steps or environmental factors in protein biosynthesis which are rate limiting (for instance, in cloudy weather when the sunlight is less strong the protein concentration in rice is enhanced; on the other hand, the total yield decreases because of an increased sterility among the plants).

A seemingly negative correlation between protein concentration and lysine concentration has been observed. Varieties with 15 percent protein content have a somewhat lower lysine concentration in their protein than those containing the more usual 7-1/2 percent protein. At present, Dr. Cruz is collecting the rising sap at the base of the rice panicle and analyzes it for total nitrogen and amino acid composition. Comparisons are made with the amino acid composition found in the final grain in an effort to establish if one amino acid is possibly the limiting factor in final protein concentration. Also, the free amino acids in the sap going into the panicle during the course of the development of the rice grain are being analyzed quantitatively to determine whether differences exist in the level of each free amino acid in low- and high-protein lines. A spectrum of high to low protein-producing rice plants is studied in this fashion. Preliminary data in selected hybrids show that the soluble amino nitrogen concentration and the rate of leucine-U-C^{14} incorporation were higher in developing grains of high protein lines than in low protein ones. Present experiments seek to determine by manipulation of N supply during grain development whether the inherent capacity for protein synthesis is dependent on the level of free amino acids in the grain. If such a relationship exists, then differences in grain protein are mainly due to varietal differences in the efficiency of roots in absorbing N from the soil as reflected in the level of free amino N translocated into the grain.

Protein efficiency ratio (PER) studies, using rats, of several parent rice strains have been carried out by Dr. Bressani at the Institute of Nutrition of Central America and Panama (INCAP) and future PER studies of the new hybrid varieties are contemplated. Results to date have shown that the growth rate of rats was more affected by differences in protein content between the samples than by differences in protein quality.

Dr. Helen Clark of Purdue University has just finished a series of human nitrogen balance studies utilizing one of the experimental high protein rice strains as the source of dietary nitrogen. This strain (BPI 76-1) was compared in adult subjects with an equal weight of rice of the commercial "Bluebonnet" variety at a daily intake of 480 grams. The experimental rice which contained 1.8 times as much protein as Bluebonnet rice, caused significantly higher nitrogen retention (P 0.01), which is attributable to the increased amounts of essential amino acids. The respective mean positive balances were 1.41 = 0.89 g and 0.24 = 0.31 g. Addition of supplementary nitrogen to make the Bluebonnet rice isonitrogenous with the BPI-76-1 variety did not cause a statistically significant improvement. All subjects were in negative balance when 320 g Bluebonnet rice were made isonitrogenous with 480 g of the same rice by adding nonspecific nitrogen.

Significance to NIAID Program and Biomedical Research: (Please see above under Objectives). Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7-1/2 percent) could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Southeast Asian populations and underdeveloped rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and of the applied nutrition program of the Institute.

Proposed Course: In the face of the encouraging progress thus far, further crossbreeding and growing of the resulting hybrids in large plots is contemplated, side-by-side with additional protein efficiency ratio determinations, in rats, and human nitrogen balance studies, of the protein of the resulting hybrid crops.

Date Contract Initiated: May 10, 1968

Current Annual Level: $30,000

MASSACHUSETTS INSTITUTE OF TECHNOLOGY (PH43-67-93) .... Malnutrition

Title: Survey of Mycotoxin Contamination of Foods and Foodstuffs in Southeast Asia

Project Director: Dr. R. N. Shank

Project Officer: Dr. John H. Weisburger

Objectives: To investigate qualitatively and quantitatively the presence of mycotoxins on foodstuffs available on the market in Southeast Asia, and particularly in Thailand. To investigate in detail the presence of aflatoxin in such foodstuffs. To develop data on the daily intake of aflatoxin and other mycotoxins per person as a function of geography. To study the possible presence of new mycotoxins with biological activity, particularly with carcinogenic potency. To relate the presence of carcinogenic mycotoxins to incidence of disease in man and animals in select regions.

Major Findings: Experimental work was completed in June 1970 and the last of the results were obtained in December 1970. Direct measurement in urban populations indicated an incidence of primary liver cancer in southern Thailand (Songkla province) of 2.0 new cases per year per 100,000 people; in the southwest (Ratburi and Nakorn Pathorn provinces) incidence was 6.0. Consumption of total aflatoxins in rural Songkla was 5 to 8 nanograms per kilogram body weight per day (on a family body weight basis), while in Ratburi it was 45 to 77 nanograms per kilogram body weight per day, 5 to 15 times greater than in Songkla. In central Thailand (Singburi province) where the incidence of liver cancer was expected to be greater than in Ratburi but could not be measured directly, consumption of total aflatoxins was 73 to 81 nanograms per kilogram body weight per day, which amounts to 20 to 30% of comparable intake values that induce nearly 100% tumor incidence in rats following continuous exposure. Because these are family averages, exposures of individual family members are undoubtedly higher. The comsumption figures do not include the amount of aflatoxin ingested with contaminated peanuts eaten as snacks. Attempts to measure liver cancer incidence by prevalence techniques were not successful because the size of the population studied (approximately 50,000 each in Songkla and Singvuri) was too small for the duration of the disease (1 to 2 months from the time of first clinical recognition to the time of death as determined in this project in a separate study of 271 cases of liver cancer). The data support the hypothesis that in Thailand aflatoxin consumption is associated with liver cancer but do not permit a definite conclusion that aflatoxin is a causative factor in the etiology of the disease. It is felt that a more direct approach to the study or a relation between mycotoxins and human liver disease is offered by the problem of Reye's syndrome in Thailand. Aflatoxin B_1 was found in autopsy specimens from 21 of 22 cases of this highly fatal children's disease.

Significance to NIAID Program and Biomedical Research: Cancer of the liver and other related debilitating diseases occur on a regional basis in many parts of the world. The exact etiology is as yet ill defined. On the basis of reasonably convincing evidence obtained in animal studies it would appear that these conditions in man may be due to consumption of food containing mycotoxins. The aim of the present effort is to secure valid and definitive information on the relationship between consumption of nutrients contaminated with mycotoxins and disease in man.

Proposed Course: The project was formally closed in December 1970 through Dr. Stand Mongkolsuk, Dean of the Faculty of Science of Mahidol University (formerly University of Medical Sciences). The Faculty is maintaining the laboratories and supporting 5 of the personnel while new funds are being sought for studies on Reye's syndrome and dietary nitrosamines and cancer of the digestive system.

Date Contract Initiated: January 11, 1967

Current Contract Level: $93,000

U.S. DEPARTMENT OF AGRICULTURE, AGRICULTURAL RESEARCH SERVICES, Interagency Agreement . . . . Malnutrition

Title: Development of Peanut and Cotton Varieties Resistant to Toxin-Producing Molds

Project Director: Dr. Robert W. Howell, U.S.D.A.

Project Officer: Dr. Benjamin T. Burton, NIAMD

Objectives: To adapt and/or develop methods suitable for the rapid and accurate assay of peanut and cotton genotypes for resistance to Aspergillus flavus and other toxin-producing molds.

Major Findings: In view of the long-range aspects of this type of investigation, the individual findings do not have too much relevance at this particular stage of the project.

Significance to NIAID Program and Biomedical Research: Malnutrition studies were one of the six specific disease categories selected for study by the U.S. Delegation to the U.S.-Japan Cooperative Medical Science Program.

Proposed Course of Project: Agreement was discontinued on December 31, 1970.

Date Initiated: April 1968.

Current Annual Level: $20,000

FOOD COMPOSITION TABLE FOR USE IN EAST ASIA . . . . Malnutrition

Amount:

Interagency Agreement with the Nutrition Program, HSMHA: $10,600

Contract for additional personnel with the American Institute of Nutrition: $34,895

Project Director: Dr. W. T. Wu Leung, Nutrition Program, HSMHA

Project Officer: Dr. B. T. Burton, NIAMD

Objectives: The primary objective of this project is the preparation of a table of food compositions for foods used in the East Asian countries (Burma, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Philippines, Taiwan, Korea, and Japan). This includes indigenous food products as well as foodstuffs imported to these countries which are consumed locally. The first Far Eastern Food Table was issued in 1945 under the auspices of the Committee on International Food Value Problems of the National Research Council, U.S.A. Although it was revised and enlarged in 1952 as Agriculture Handbook No. 34, "Composition of Foods Used in Far Eastern Countries", it is not applicable to conditions in 1969. Comprehensive food tables for local foods in East Asia are essential for and basic to any attempt of improving the nutritional state of East Asian populations through a combination of nutrition education or nutrition improvement through introduction of innovations in the local food consumption.

Major Findings: The Principal Investigator, Dr. Leung, who was responsible for the preparation of the first Far Eastern Food Table immediately after World War II, is collecting all available food data through correspondence and through personal visits in the various countries. In addition, there is active collaboration with the Nutrition Division of the Food and Agriculture Organization of the United Nations, through Dr. K.K.P.N. Rao and his staff who have accumulated a considerable body of data at FAO Headquarters in Rome and are constantly generating new information in regional FAO offices in Asia.

The information collected includes: data on moisture, "proximate composition", minerals, vitamins, amino acids, fatty acids, trace elements, refuse, and local methods of preparation, processing and preservation of foods, etc. Whenever feasible, local names used in various Far Eastern countries with the corresponding English and scientific names are recorded and collected. The result of this project will be printed as a joint publication "Food Composition Table for Use in East Asia" - NIAMD will be responsible for the printing of the English version, and FAO will undertake the translation and printing in French. The format of the food table will follow closely the one previously designed for Africa.

Data collection was begun in the winter of 1969-70 and is progressing very satisfactorily.

Significance to NIAID Program and Biomedical Research:
Please see "Objectives".

Proposed Course of Project: It is expected that data collection will proceed for about two years while, simultaneously, tables will be coordinated and assembled in Bethesda. A third year will be necessary for the final organization, editing and printing of the publication.

Date Initiated: A.I.N. - January 29, 1970
HSHMA - October 15, 1969

Current Annual Level: A.I.N. - $35,000
HSHMA - $10,000

UNIVERSITY OF AKRON (NIH-70-2273)........Parasitic

Title: "Development of a Toxic Bait for Destruction of Biomphalaria glabrata and Other Snail Vectors of Schistosomiasis"

Project Director: Mr. Nathan Cardarelli

Project Officer: Dr. Kenneth Phifer

Objectives: To formulate and manufacture on a pilot scale a toxic bait for the control of the snail vectors of schistosomiasis. The toxic bait combines the slow release of a snail attractant with a molluscicide which kills the snail following ingestion. Those formulations which have been effective in laboratory screening will be evaluated in field test sites under a variety of environmental conditions.

Major Findings: Several promising formulations have been developed which, on laboratory screening, appear to be quite effective in killing snails under a variety of experimental conditions (wide pH range, running water vs. static conditions, etc.). Wheat germ has proven to be the best attractant. Both Bayluscide and Frescon have been bound into the pellet and are effective at low concentrations with respect to molluscicidal activity. The program has developed to a point where it is now feasible to field test a series of combinations of baits, molluscicides and acrylic binders against Biomphalaria glabrata, the major vector of schistosomiasis.

Significance to NIAID Program and Biomedical Research: Control of the snail vectors of schistosmiasis is highly relevant to the mission of the U.S.-Japan Program. There is great need for the development of molluscicides with residual activity since biological control of these vectors is still a goal of the future.

Proposed Course of Project: The immediate aims of the project are expected to be reached in one year at which time the project will be reviewed and considered further development.

Date Contract Initiated: June 26, 1970

Current Annual Level: $17,296

UNIVERSITY OF CALIFORNIA AT LOS ANGELES (NIH-70-2009).......Parasitic

Title: "Supplying Genetic Marker and Incompatible Strains of Culex pipiens"

Project Director: Dr. A. Ralph Barr

Project Officer: Dr. Kenneth O. Phifer

Objectives: To breed and to maintain stocks of Culex pipiens mosquitoes for work in filariasis research. These stocks have been available for use by competent investigators upon request.

Major Findings: Six new strains were added during the past year and several of the strains being maintained were improved. Inheritance of characters was studied with emphasis on writing computer programs for the estimation of crossover rates.

Significance to NIAID Program and Biomedical Research: Culex pipiens is the most important vector of human filariasis in the world. This mosquito has been very difficult to control by conventional methods. This source was developed to stimulate increased work on the basic genetics of C. pipiens which can then be applied in effective programs of biological control.

Proposed Course of Project: This project will be discontinued since requests for material have not been sufficient to justify its extension.

Date Contract Initiated: April 23, 1970

Current Annual Level: $15,321 (prior year support)

UNIVERSITY OF GEORGIA (NIH-69-92)......Parasitic

Title: "Filariasis Chemotherapy and Repository" - Research Services

Project Director: Dr. Paul E. Thompson

Project Officer: Dr. Kenneth O. Phifer

Objectives: To evaluate selected compounds for antifilarial activity in experimental animals; to maintain colonies and life-cycle of vectors and parasites for four species of filaria to be used in medical research; to supply qualified investigators with filariasis research materials in response to authorized requests.

Major Findings: Fifty-eight different drugs have been tested for their chemotherapeutic activity against filariasis. Approximately four shipments per month were made to provide materials for individuals investigating a variety of problems in filariasis. During the past year, 19 new requestors have utilized the contract and have thus become involved in filariasis research.

Significance to NIAID Program and Bio-Medical Research: Basic research in filariasis has been retarded by the difficulty inherent in the maintenance of the life cycles of filarial parasties. The supply of filarial material has encouraged immunologists, biochemists, pharmacologists, etc., to undertake needed studies in this area. In addition, there has been a need for an intensified search for better chemotherapeutic agents. The contractor has been able to apply both his expertise and his laboratory facilities to this aspect of filariasis research.

Proposed Course of Project: This project will be funded as long as there is a need for the services provided.

Date Contract Initiated: January 22, 1969

Current Annual Level: $55,215

UNIVERSITY OF MICHIGAN (PH-43-67-736)........Parasitic

Title: "Supplying Animals Infected with Three Human Schistosomes"

Project Director: Dr. Elmer Berry

Project Officer: Dr. Kenneth O. Phifer

Objectives: To maintain colonies and life-cycles to supply parasites, infected animals and intermediate hosts of the three human schistosomes and other schistosome materials to selected investigators.

Major Findings: In the past year 130 shipments were made to 34 investigators. Of these investigators, 14 were utilizing the supply contract for the first time. Included in the 130 shipments were 4869 infected rodents and 3735 infected snail vectors. In addition, the Contractor's staff provided short-term training in routine procedures of snail maintenance to investigators from seven countries.

Significance to NIAID Program and Bio-Medical Research: The supply of this material enables a number of qualified investigators to carry out research in the field of schistosomiasis. If each investigator had to maintain complete life cycles of this parasite, very few laboratories would be engaged in Schistosomiasis research. Since this disease is second in terms of world-wide morbidity, infecting over 200 million people, research stimulated by the availability of schistosome material is definitely of biomedical significance.

Proposed Course of Project: To continue to supply schistosome material to investigators now working in the field and to stimulate additional investigators to enter this area of parasitological research.

Date Contract Initiated: April 12, 1967

Current Annual Level: $63,000

STANFORD RESEARCH INSTITUTE (NIH-71-2122)........Parasitic

Title: "Synthesis of Labeled Diethylcarbamazines"

Project Director: Mr. Morris Leaffer

Project Officer: Dr. Kenneth O. Phifer

Objectives: To prepare three separate isotopically labeled diethylcarbamazines. These products are to be prepared in three-gram quantities each and packaged in 100 mg lots. The products will be distributed to competent investigators upon request.

Major Findings: This is a new contract; no progress has been reported to date.

Significance to NIAID Program and Biomedical Research: There is a dearth of knowledge in the field of filariasis chemotherapy. Much work needs to be done in the metabolism of diethylcarbamazine, the current drug of choice, since little is known concerning its mechanism of action. This source of isotopically labelled drug will enable investigators to elucidate the metabolic fate of diethylcarbamazine and to make possible the synthesis of analogues leading to more effective antifilarial compounds.

Proposed Course of Project: The Project will remain in force as long as there is a demand for these products.

Date Contract Inititated: March 22, 1971

Current Annual Level: $6,922

HAZLETON LABORATORIES (NIH70-2242) ... Tuberculosis

Title: Study of BCG Vaccination of Subhuman Primates

Project Director: Dr. Robert C. Good

Project Officer: Mr. George S. Yee

Objectives: To develop a standardized test method in subhuman primates for the evaluation of tuberculosis vaccines.

Major Findings: The contractor has completed vaccination and aerosol challenge of various animal groups. Data are being compiled on skin tests, serial x-ray studies and serological investigations.

Significance to NIAID Program and Biomedical Research: Because of variations in methodology, it has not been possible to compare vaccination test results from one laboratory to those of another laboratory. It is important to devise a standardized test method for the evaluation of tuberculosis vaccines.

Proposed Course of Project: This project will be funded for one year or until completed.

Date Contract Initiated: June 1970

Current Level of Funding: Approximately $96,200

NATIONAL JEWISH HOSPITAL (NIH 70-2079) ..... Tuberculosis

Title: Serological Identification of "Atypical" Mycobacteria

Project Director: Dr. Werner B. Schaefer

Project Officer: Mr. George S. Yee

Objectives: To maintain reference cultures and type-specific antisera to "atypical" mycobacteria. To serve as a reference laboratory for serological identification of "atypical" mycobacteria.

Major Findings: In addition to the distribution of reference strains and corresponding antisera to "atypical" strains of M. avium, M. intracellulare amd M. scrofulaceum, the Project Director has been able to separate serologically the photochromogenic species, M. Kansasii and M. marinum. He also showed that M. fortuitum, M. abscessus and M. peregrinum (species with similar cultural characteristics) can also be identified by agglutination and/or agglutinin absorption tests. Another significant finding was that scotochromogens are antigenically heterogeneous and cannot be identified serologically. A study is underway to determine whether antigenically heterogeneous strains of M. marinum can be divided into well-defined subtypes.

Significance to NIAID Program and Biomedical Research: Studies of "atypical" mycobacteria isolated from human infections suggest that these infections have a common source; therefore, the maintenance of a reference laboratory will serve those engaged in mycobacterial research.

Proposed Course of Project: This project will be continued as long as there is a need for the services required.

Date Contract Initiated: February 17, 1970

Current Level of Funding: $18,000

TRUDEAU INSTITUTE, INC. (PH 43-68-640) . . . . Tuberculosis

Title: Authentication, Maintenance, and Distribution of Mycobacterial Cultures.

Project Director: Dr. George P. Kubica

Project Officer: Mr. George S. Yee

Objectives: To characterize, distribute and maintain "prototype" mycobacterial strains.

Major Findings: Approximately 200 strains of mycobacteria have been authenticated both *in vitro* and *in vivo* by growth characteristics, animal pathogenicity, drug susceptibility, and other biological parameters. One-hundred thirty cultures have been characterized and packaged in 7H-9 Middlebrook media for storage at -70°c and about 400 cultures have been distributed to 72 requestors. A catalog of available strains is being prepared.

Significance to NIAID Program and Biomedical Research: This culture collection, when characterization studies are completed, will provide a source of standardized research material to those engaged in various aspects of research involving mycobacteria.

Proposed Course of Project: The contract will be continued for the characterization of existing strains. Other strains of interest will be characterized and added to the collection for taxonomic purposes.

Date Contract Initiated: March 25, 1969

Current Annual Level: $55,870

UNIVERSITY OF WISCONSIN (NIH 69-2175) .... Tuberculosis

Title: Development of a Test System for Evaluating Tuberculosis Vaccines

Project Director: Dr. Donald W. Smith

Project Officer: Mr. George S. Yee

Objectives: To develop a standardized test methodology by which tuberculosis vaccine potency can be determined before these vaccines are subjected to clinical trials.

Major Findings: The contractor has utilized a multivariate analysis which includes an orthogonal, individual degrees-of-freedom technique. It is hoped that this systematic examination of test systems will identify those variables that may influence the ranking of potencies of tuberculosis vaccines.

Significance to NIAID Program and Biomedical Research: Because of variations in methodology, it has not been possible to compare vaccination test results from one laboratory to those of another laboratory. It is imperative that a standardized test method be devised for the evaluation of tuberculosis vaccines.

Proposed Course of Project: This project will be funded for an additional year or until completed.

Date Contract Initiated: June 1969

Current Annual Level: $45,000

Title: Work on an Improved Method for Inactivation of Rabies Virus Without Significant Loss of Immunogenicity

Project Director: Dr. Kenneth Wilcox

Project Officer: Dr. Kenneth O. Phifer

Objectives: To develop a safe and effective procedure for the inactivation of rabies virus. The inactivating agent, beta-propiolactone, will be compared with formalin in determining the kinetics of rabies virus inactivation and the effect of the agent on rabies virus antigen. To test for completeness of inactivation and residual levels of inactivating agent.

Major Findings: None at this early stage.

Significance to NIAID Program and Bio-Medical Research: Current rabies vaccines do not produce reliable protection and often evoke paralytic accidents. Thus, there is a need for an improved, human post-exposure rabies vaccine which produces strong and lasting immmunity in a short period of time after a minimum of injections. One aspect of the development of an improved vaccine involves the improvement of methods of inactivation of rabies virus without a loss of immunogenicity.

Proposed Course of Project: The aims of the project are expected to be reached in two years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 1971

Current Annual Level: $46,873

Title: Isolation and Characterization of Protective Antigens of Rabies Virus

Project Director: Dr. Edward Eckert

Project Officer: Dr. Kenneth O. Phifer

Objectives: To isolate, concentrate and identify the essential protective antigens of rabies virus in order to obtain a highly refined and more effective post-exposure vaccine for use in man. This will involve the isolation and characterization of protein and glyco-protein sub-units of rabies virus. Another objective of this study will be to evaluate the antigenic potency of subunit rabies vaccines and the role of the physical state of the antigens and adjuvants on immune response.

Major Findings: This is a new contract; no progress has been reported to date.

Significance to NIAID Program and Bio-Medical Research: One of the principal objectives of the U.S.-Japan Program is the encouragement of research on rabies with emphasis on studies leading to an improved post-exposure rabies vaccine. The standard rabies treatment involves a long series of inoculations of vaccines which do not provide reliable protection and which may evoke paralytic accidents. One of the most relevant areas of endeavor is the isolation and identification of the essential protective antigens or rabies virus with the ultimate objective of developing a clean vaccine which produces strong and lasting immunity in a short period of time after a minimum number of injections.

Proposed Course of Project: The immediate aims of the project are expected to be reached in three years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 1971

Current Annual Level: $20,616

UNIVERSITY OF TEXAS MEDICAL SCHOOL AT SAN ANTONIO (NIH 69-2049)..Viral Diseases

Title: Purification of Rabies Vaccine by Exclusion Chromatography on Glass of Controlled Pore Size

Project Director: Dr. C.W. Hiatt

Project Officer: Dr. Kenneth O. Phifer

Objectives: To devise a commercially feasible method for purifying rabies vaccines of brain origin by removing the specific impurities which cause allergic encephalitis. To develop and improve the technology or virus purification by steric exclusion chromatography on porous glass.

Major Findings: Operating conditions for vaccine purification were adjusted by using a mixture of substances simulating the essential physical properties of rabies vaccine. Experimental allergic encephalitis was produced in guinea pigs in a study of positive control animals. Techniques were devised for measurement of residual polyethylene oxide (an additive used to prevent adhesion of virus to glass). Several experiments have been carried out in which infected brain tissue has been placed on the glass bead column. Viral material was separated from brain tissue. Currently, "purified" virus material is being inactivated in preparation for pilot vaccine studies.

Significance to NIAID Program and Bio-Medical Research: Present rabies vaccines contain undesirable constituents which are not part of the virus. This technique has proven effective in the purification of other virus and thus should prove equally fruitful with rabies virus. In addition, since the use of brain tissue vaccines is still being widely used in Asia, this project is quite relevant to the mission of the U.S.-Japan Program.

Proposed Course of Project: This project will be extended until December 1971 to enable the contractor to complete his studies.

Date Contract Initiated: May 13, 1969

Current Annual Level: $40,544 (prior year support)

THE WISTAR INSTITUTE (NIH 71-2292)..................Viral Diseases

Title: Improved Methods for Potency Testing of Standard and Refined Rabies Vaccines

Project Director: Dr. Tadeusz Wiktor

Project Officer: Dr. Kenneth O. Phifer

Objectives: To develop simple and economical procedures for the evaluation of the protective activity of rabies vaccines by means of an in vivo test in which treatment will follow the virulent virus challenge. To develop an in vitro procedure for a rapid determination of the protective activity of such vaccines. To determine which of the presently available serological assays for rabies antibody best measures the state of resistance to rabies infection.

Major Findings: None at this early stage.

Significance to NIAID Program and Bio-Medical Research: The U.S.-Japan Program considers relevant the encouragement of research on rabies with emphasis on studies leading to an improved post-exposure rabies vaccine since currently available vaccines represent one of the crudest and most unsatisfactory biological products in use in man. One aspect of the development of an improved post-exposure vaccine involves the improvement of methods of potency testing of standard and refined vaccines since existing procedures do not adequately characterize vaccines of tissue culture origin.

Proposed Course of Project: The immediate aims of the project are expected to be reached in from two to four years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 1971

Current Annual Level: $35,933.40

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EXTRAMURAL PROGRAMS - NIAID

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ANNUAL REPORT OF PROGRAM ACTIVITIES

National Institute of Allergy and Infectious Diseases
July 1970 - June 1971

I. ACTIVITY REVIEW

Research Grants

The NIAID has continued to maintain a high quality research grants program during FY 1971. Sixty percent of the research grants program budget is expended in support of research projects in the immunology, microbiology and parasitology disciplines. These fundamental research projects are carefully selected for relevance to the NIAID mission as well as for scientific merit. Forty percent of the research grant support is awarded in support of special emphasis programs. These programs are also composed of investigator-initiated projects chosen, and frequently programmed, by staff because they will further specific goals of the special emphasis programs of the Institute. These programs are reviewed regularly by NAAID Council, and their selection, development, and eventual phasing down is carried out with Council advice. During FY 1971, the NAAID Council selected the subject "Immunity to Animal Parasites" as a special emphasis program. This decision represented an opinion that there were now special opportunities to control parasitism in animals and man through research on the immune mechanism.

A Fiscal Year 1971 regular research grant budget increase of 3% over Fiscal Year 1970 was offset by a steady increase in average direct and indirect grant costs, decreased savings from prior year grant budgets to apply to current year awards, and an increase in number and amount of applications approved for funding. The Extramural Programs staff, responding to an NIH directive, negotiated an overall 5% reduction in grant budgets in addition to normal savings and policy restrictions. This reduction enabled the Extramural Programs to support 50 approved high priority applications which would have not otherwise been funded. Approximately 50% of the applications reviewed and approved by the National Advisory Allergy and Infectious Diseases Council for FY 1971 support were funded. However, this represented a smaller number of research grants than were supported in FY 1970 when an average 9% across-the-board reduction was made in all types of research grants.

An Allergic Disease Centers program was established, with initial funding during FY 1971 of seven centers. Savings from the existing Categorical Clinical Centers program and transfers from training grants and fellowships provided support for these projects.

Training Grants and Fellowships

A 30% reduction in funds available to award approved training grant applications in FY 1971 compared to FY 1972 required that extramural staff continue its strict review of application budgets, limiting amounts awarded for staff salaries, equipment, supplies and staff travel. Approximately 37% of approved applications were funded.

A transfer of $170,000 was made from fellowships to the Categorical Clinical Centers program in FY 1971 to provide additional resources for the new Allergy Disease Center activity. Funds available for fellowships allowed award of approximately 55% of approved postdoctoral, special, and research career development award applications. The total number of postdoctoral and special fellowship applications increased 75% in FY 1971 over the preceding year.

The Training Grant Committee assumed responsibility for initial review of postdoctoral and special applications, while relinquishing review of research career development awards to the Division of Research Grants study sections.

The Training Grant Committee recommended that special emphasis areas be identified for consideration in awarding training grants. Six areas considered for such designation were pathogenic microbiology, antimicrobial agents and drug resistance, mycology, allergy and clinical immunology, immunogenetics, and host-parasite relationships. The recommendation included methods to encourage support within these special interest fields. These suggestions are under consideration by the NAAID Council and a decision on designating special emphasis areas for the training program must await Council review of the special emphasis program in the research grants program.

Council Subcommittee on Criteria

A Systems/Operations Analysis of Programs (SOAP) was presented by the National Bureau of Standards for National Advisory Allergy and Infectious Diseases Council consideration. This study determined that criteria other than scientific merit was appropriate, but often difficult to delineate, in the review and payment of research applications. Council directed that staff develop methods for determining payment levels for special emphasis areas according to specific allocations of available funds. Staff was also asked by Council to provide new definitions for special emphasis area programs.

The Council recommended that the Institute accept the concept of the dollar allocation to program areas of special emphasis rather than adjusting priority scores in order to fund projects assigned to special emphasis areas. Methods for application of this "dollar allocation concept" will be developed gradually during the coming fiscal year. Staff will also be developing the methods for providing Council the information necessary to make decisions on adjusting budgets in special emphasis programs and initiation of new programs.

Personnel

The Extramural Programs was allowed two additional positions during FY 1971. One of these positions was assigned to an employee from the Career Development Review Branch, DRG, when the fellowship review activity was decentralized. This employee has been of considerable assistance in managing the review of fellowship applications and assisting with training grant activities. The other position was used to recruit a medical officer to assist with the development of our clinical programs, including the clinical allergy centers.

The attached graph illustrates the steady decline in the number of personnel positions assigned to the Extramural Programs since 1966. The graph illustrates that the number of grants awarded and the number of grants reviewed has remained relatively constant. It does not show the increased workload assigned to the Extramural Programs by budget negotiations begun in FY 1969, by additional correspondence and grantee contacts as a result of requests for information about grant applications not paid, or by increased administrative actions such as reviews for patient experimentation and FAST Committee investigations. Many clerical activities of the Extramural Programs, such as secretarial assistance for the Training Grants Officer, Program Analysis Group, assistance in preparation of Council material, Mail-File Room, and duplicating activities, are maintained by temporary and part-time employees.

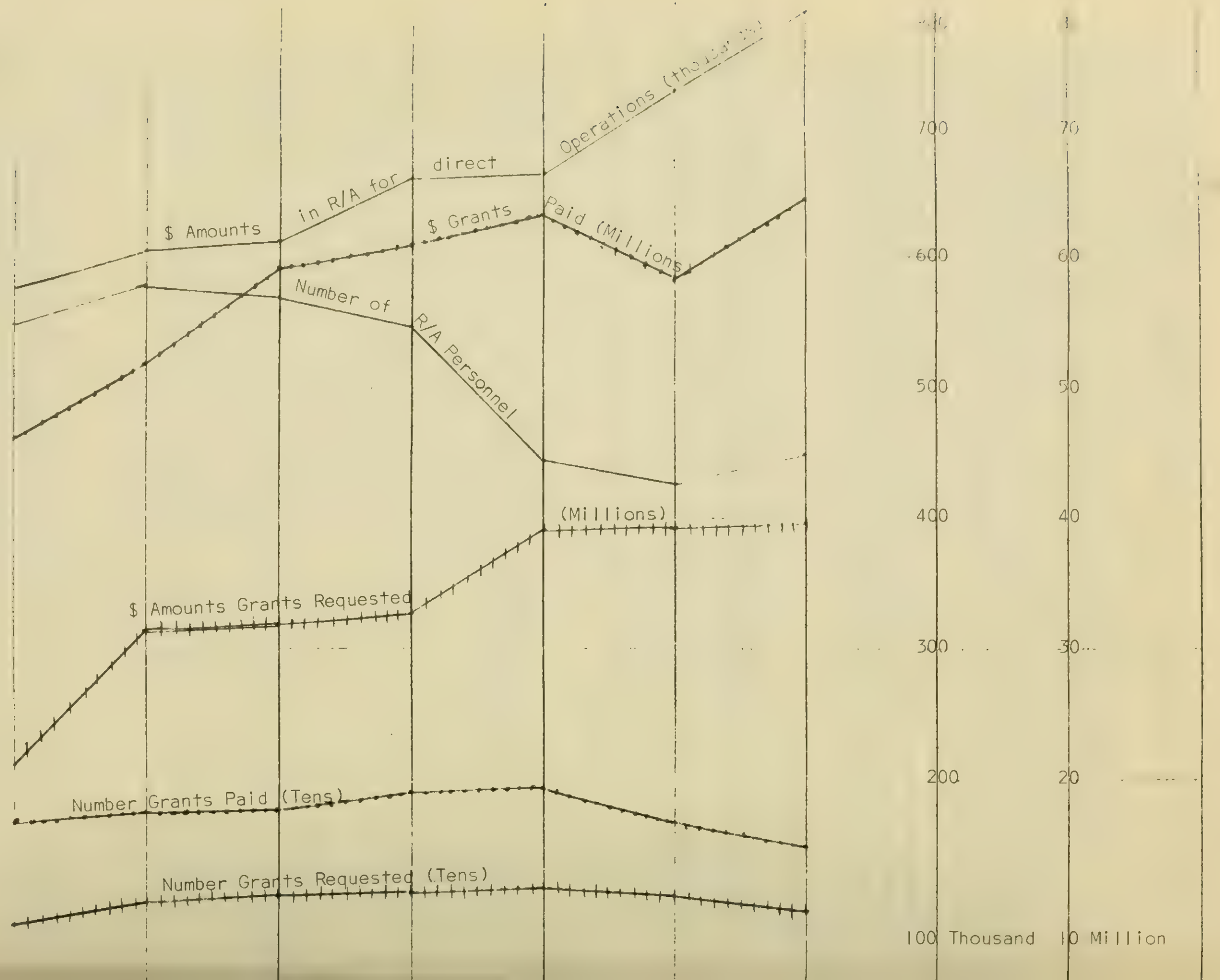
Personnel Changes

1. Dr. Francis R. Abinanti, former Associate Director for Extramural Programs, was appointed to the intramural laboratory staff, NIAID, September 1, 1970.

2. Dr. William I. Gay was appointed Associate Director, Extramural Programs, NIAID, December 13, 1970.

3. Dr. Robert T. Scholes, Assistant Associate Director, Extramural Programs, retired June 1, 1971.

4. Dr. Luz Froelich was appointed Assistant to the Associate Director for Clinical Programs, June 14, 1971.


$ Amounts in R/A for direct Operations (thou
$ Grants Paid (Millions)
Number of R/A Personnel
(Millions)
$ Amounts Grants Requested
Number Grants Paid (Tens)
Number Grants Requested (Tens)
700
600
500
400
300
200
100 Thousand
70
60
50
40
30
20
10 Million

National Advisory Allergy and Infectious Diseases Council

Members of the Council for the period ending June 30, 1971, were:

Dr. Elisha Atkins
School of Medicine
Yale University

Dr. Wilford S. Bailey
Auburn University

Dr. J. Werner Braun
Rutgers, The State University

Dr. C. West Churchman
University of California

Dr. Leighton E. Cluff
University of Florida

Dr. Irwin C. Gunsalus
University of Illinois

Dr. Charles A. Janeway
Children's Hospital Medical Center

Dr. Thomas F. Paine
Nashville General Hospital

Dr. Frank A. Sloan
RAND Corporation

Dr. Carlos N. Vicens
City of San Juan, Puerto Rico

Dr. Duard L. Walker
The University of Wisconsin

Mr. William White
Family Health Magazine

Ex officio members:

Dr. James H. Matthews
Veterans Administration

Capt. Robert O. Peckinpaugh
Great Lakes Naval Hospital

II. PROGRAM REVIEWS

A. ALLERGY AND IMMUNOLOGY

The approximate level of support given allergy and immunology:

Type of Grant	Number	Amount
Research grants	261	$12,316,000
Training grants	41	2,631,000
Fellowships	53	1,038,000

Summary

Developments in immunology during the past year have again been largely dominated by investigations at the cellular level. The extremely varied spectrum of issues in cellular immunology all continue to be heard from, and progress is evident in virtually every direction. However, from the long-range point of view, there are three areas in which there were developments of singular import: (1) cell surface antigen receptor sites and neoplastic transformation; (2) Epstein-Barr (EB) virus in the human environment and its intimate interactions with lymphocytes; (3) Lymphocyte membrane markers. Developments in these three areas involve the most fundamental issue of the immunology of cell membrane structure, its modulation, and its critical role in the regulatory processes presumed to be operative in the complex host environment. Developments in these areas may be detailed as follows:

(1) Concanavalin A is one of a group of phytoagglutinins or lectins, glycoproteins isolated from various plant seeds, which bind and agglutinate animal cells and are extremely effective in activating or triggering lymphocytes *in vitro*. Concanavalin A is directly related to the fact that transformed cells are not subject to contact inhibition of cell division. Thus, cells transformed by polyoma virus always bind Concanavalin; if the sites which bind this mitogen are first covered by an enzymatic fragment of Concanavalin, contact inhibition is restored and these cells are now indistinguishable from normal cells in their interactions. The restoration of growth control by the enzymic fragment of Concanavalin A is fully reversible. When the agglutinin is removed from resting transformed cells, they start to divide once more. From these data, the picture that emerges is that the immune receptors for the phytoagglutinin are part of an entire subsurface layer in mammalian cells. In untransformed cells, this layer is covered by some thus far uncharacterized substance or cell architecture; in transformed cells or in trypsin treated normal cells, the subsurface layer is respectively permanently or transiently exposed. It thus seems evident that cells can only propogate and/or respond to short-range chemical messages which lead to a cessation of cell division when the subsurface layer

containing the phytoagglutinin receptors is masked. When the agglutinin receptor layer is exposed, cells do not seem to be able to recognize and respond to the near presence of other cells.

(2) The detection of intracellular herpes-like EB virus and various EB antigens in established lymphoid suspension cultures from peripheral blood of patients with malignant and benign lymphoproliferative states and from clinically healthy subjects as well, has led to the speculation that a virus endowing human cells with oncogenic potential both _in vivo_ and _in vitro_ has at last been uncovered. Seroepidemiologic surveys confirm that the EB agent is ubiquitous in the general population and detailed analysis of the incidence and distribution of EB antibody titers has now implicated this agent in the pathogenesis of several benign and malignant proliferative disorders of unknown etiology; for example, infectious mononucleosis and sarcoidosis. Patients with well defined viral diseases have shown that the EB virus, as detected by long-term lymphoid cell culture, exists in some individuals in whom no anti-EB antibody can be measured by standard screening tests. Seroconversion to the EB virus in such individuals already harboring the virus in an intracellular compartment must therefore depend on factors other than primary exposure to this ubiquitous agent. The presence of detectable EB IgM antibody in patients acutely ill with infectious mononucleosis and the subsequent IgG anti-EB antibody response suggests that this may be an anamnestic reaction. The presence of this herpes-like virus in most lymphoid cell lines derived from normal individuals further suggests (a) that this agent is a latent virus which may be essential for proliferation of lymphoid cells in long-term culture, or (b) that the virus may simply be activated by proliferative events affecting the host cell _in vivo_ and _in vitro_. The role of cellular immunity in host reactions to EB infection is only now beginning to receive attention. Cell mediated immunologic responses appear to be the primary means by which the immunocompetent host restricts intracellular parasitism by microbial agents and maintains surveillance for neoplastic cells. Cell-mediated immunologic reactivity appears to be undiminished in infectious mononucleosis, where the anti-EB titers tend to be low. Infectious mononucleosis may thus represent a natural model for virus associated transformation and virus targeted immunologic surveillance. This is, in fact, attested to by the now confirmed findings that the derived established lymphocytic cell lines are actually antigenic when set up in mixed lymphocyte cultures with the cells of the autochthonous host. The target is in all probability a virus-directed membrane component.

(3) Membrane markers are becoming increasingly important for the identification of different populations of lymphoid cells and are now used in a sophisticated way to clarify the complex series of cellular events in the immune response. There are three basic categories of markers on lymphocyte membranes of most species: (a) alloantigens; (b) immunoglobulin determinants; and (c) receptors for complement components. It is especially significant that there is now accumulating evidence that these constitute three distinct populations of cells having characteristic physical properties. The distribution of cells with each of these distinctive markers differs widely in various lymphoid organs. These specific markers on lymphoid cells are probably the result of differentiation, by selective activation of genes characteristic of these cells, and is almost certainly related to their function. The life history, fate and function of these immunocompetent cells, expressed as specific cell contacts, tissue organization, and the stimulation of repression of unique activities are likely to be regulated or triggered by interaction of membrane recognition units. It seems likely that this basic line of investigation will dominate research in immunobiology for many years to come.

ALLERGIC DISEASE CENTERS PROGRAM

During this period, plans for an Allergic Disease Centers program were formulated, the means for its funding was determined, and the beginning of a national program was brought to fruition. This activity had its beginnings in discussions in which it was decided that substantial savings could be effected in ongoing large transplantation center grants and that such funds could be utilized to advantage for initiating the development of new programs in clinical allergy. It was felt that the NIAID commitment to the area of clinical immediate hypersensitivities states such as the pollenoses, sensitivities to insect stings, food and drug sensitivities, and asthma, to name but a few, known to affect a large proportion of the population, was neither as large nor as effective as desired. This unsatisfactory state in an important field was in contrast with the rapid and spectacular advances in most key areas in the discipline of immunology, notably in the delayed hypersensitivities of cellular immunities. There are good and sufficient reasons for this imbalance, but this in no way diminished the NIAID concern and desire to encourage, support and facilitate progress in dealing with human problems in the immediate hypersensitivities.

It was therefore decided that with the extremely limited fiscal resources that could be marshalled, the most constructive action the Institute could take was to provide support for a number of the major investigators in this area who had, on their own initiative, developed a solid laboratory research program that was yielding leads sufficiently encouraging as to warrant exploration for effecting a transition from laboratory findings to clinical trials. Discussions were held with national leaders in this field and reviewed issues ranging from improved diagnosis and prophylaxis to various therapeutic measures involving immunopharmacologic agents, antisera, modified allergens, etc. All consulted were enthusiastic over the prospect and appreciative of the Institute's initiative in an important and timely enterprise. It was then decided to bring the program to the attention of allergists nationally so as to provide an equal opportunity to compete for the limited funds available. Accordingly, on August 6th, an announcement to all major medical institutions was sent out. Inquiries in response to the announcement were replied to by telephone and letter, emphasizing the distinctive character of the program and the special needs. A large proportion of the letters of intent and inquiries involved groups who did not qualify, and consequently many were not encouraged to submit formal applications.

In recognition of the distinctive character of these clinically-oriented programs, special arrangements were made for the review process. The AI Branch made recommendations regarding qualified reviewers and DRG established a Special Study Section in which was represented basic immunology, clinical allergy, pharmacology and respiratory physiology, to assess the total of 18 applications that were received by the deadline of November 25th. Reviewers met in Bethesda on January 18th, approved ten applications with priority scores ranging from 128 to 367, and disapproved the remaining eight. Subsequently the NAAID Council concurred in the funding of six and, following two site visits, recommended funding of the seventh. Accordingly, this national program initially embracing seven centers will begin operations later in 1971.

PERSPECTIVES IN IMMUNOLOGY - NIAID SYMPOSIA

This series of international immunology conferences, held at Brook Lodge, has now advanced considerably towards realization of the ambitious goals set some time ago for this experiment in communication. A series of five conferences has been projected, and the fourth of these on "Immunologic Intervention" was recently held on April 26-28, 1971. Three volumes recording edited versions of past conferences have already been published by Academic Press, the most recent addition to this series being the one on "Immune Surveillance", issued in February 1971.

The series is now solidly established as a significant force in the field of immunology. The publisher has given the volumes the benefit of extensive advertising; they have had a number of good reviews in professional journals and have been favorably commented on by the immunology community. They are now regarded as a distinctive contribution to the immunology literature and have helped to consolidate NIAID as the NIH Institute most directly associated with basic research in immunology.

The recent fourth conference on Immunologic Intervention was in several respects a challenging and novel departure from the preceding specifically focused fields selected for development in that it was sought to integrate basic immunological concepts with approaches to clinical problems. Several considerations led to this choice of subject, including the recognition that the immunological literature already had an abundance of information relevant to the potential solution of many clinical problems actually a good deal more than most immunologists seem aware of. Moreover, information is rapidly accumulating in several new areas - of a kind especially promising for direct clinical application. It was also felt that most investigators have now become cognizant of the importance of bringing to bear individual and collective efforts towards utilizing the basic immunologic knowledge so painfully acquired.

Among the major areas dealt with by the conference were: reconstitution of immune responsiveness by bone-marrow, thymus grafts, and by transfer factor; stimulation of selected subpopulations of lymphocytes by alteration of immunogen or immunization regimen; deletion of selected subpopulations by induction of tolerance or administration of antisera directed against surface antigens on such subpopulations; immunosuppression by passive antibody; removal of immunocompetent cells, antibody and other molecules from the circulation by columns of immunoadsorbents; use of antibody conjugated to toxic agents selectively to destroy cells or tissues; and new approaches to immunotherapy of cancer.

SCIENCE WRITERS SEMINAR ON IMMUNOLOGIC INTERVENTION

A science writers seminar was organized and held in Atlantic City shortly after the fourth Brook Lodge conference in order to brief a specially selected group of eight experienced writers reporting for major newspapers, weekly news magazines and key medical news publications. Eight of the 36 Brook Lodge conferees met informally with the science writers on May 1 and 2 for a total of approximately five hours and conveyed to them the highlights of what in their individual and collective opinions represented some of the noteworthy developments that emerged from the Brook Lodge conference. The role of NIAID in the organization of the overall enterprise was identified and the entire subject of manipulation of the immune response and its implications for immunotherapy was reviewed. The principal theme of the seminar was to substantiate with examples the message that basic immunologic knowledge had advanced to the point where it was now possible in a great variety of ways deliberately to modify, alter or manipulate the immune response. It was pointed out that these developments have important implications for clinical medicine. Additionally, it was made clear to the writers that the conference was considered notable and significant in exemplifying the fact that investigator-initiated grant supported research had the capability of solving practical problems.

NEW IMMUNOLOGY PROGRAMS

Clinical Immunology Units

There is emerging a rapidly growing appreciation of clinical immunology as a distinctive specialty, paralleled by an awareness that its representation in an organized form is becoming critically essential at centers of academic medicine. The past decade has seen a progression of clinical disease states of uncertain etiology come to be identified as immunopathologies. This unfolding development is still far from complete. While precise statistics on incidence and distribution are accumulating slowly, it is the opinion of experts that the frequency of these clinical situations may be the order of one in five, i.e., 20% of hospitalized patients (other than those with acute illnesses) are believed to have some immunologic derangement. Exclusive of allergies (the immediate type hypersensitivities), this is considered to involve more than ten million people who suffer from immune complex diseases like arthritis, Lupus erythematosis or amyloidosis.

These situations reflect for the most part either genetic defects or abnormalities, autoimmune states, or the long-term consequences of chronic viral or microbial infection. The informational base and consequent means for diagnosing and treating these clinical syndromes has advanced swiftly, but in practical terms, these resources are still conspicuously absent at most centers of academic medicine. Despite this need and the adequacy of present knowledge, present circumstances make it evident that clinical immunology resources, even at major medical centers, are likely to develop at a rather slow pace. In order to speed up this transition and to effectively provide improved diagnosis and treatment of immunologic diseases, and thereby greatly enhance delivery of essential health services, a number of factors are required: (a) the training of clinical immunologists - this has been and continues to proceed apace via NIAID immunology training grants; (b) definition and expansion of the analytic tools of clinical immunology - this has been fostered by NIAID by means of organizing workshop-conferences on methodology. The first of these on "In Vitro Methods of Cellular Immunity" was held in June 1970, and the authoritative compendium on these essential procedures was published in April 1971 by Academic Press; (c) organization of clinical immunology units - eight national experts in clinical immunobiology were consulted informally for the purpose of determining whether there is a proper role for NIAID whereby the Institute could help facilitate the establishment of such units. There was a clear consensus that there was indeed a vital role the Institute could fill. The essential requirement was considered to be a core of faculty to represent basic immunology and thereby assure an intellectual nucleus for the activity which would most likely be associated with departments of medicine or pediatrics. To assure development and growth of such a program nationally, the view was that model units should be established at ten medical schools. There was concurrence that support in the amount of $50,000 each might suffice to provide the base (supplemented by matching funds) to cover the salary for one senior and two junior faculty.

Experts are convinced that NIAID would in this manner make a major contribution to the emergence of clinical immunology as a timely and very special means of assuring improved health care. A "position paper" and recommendations are being drafted by the group for Institute guidance and for information of NAAID Council.

Program Projects in Lymphocyte Biology

Developments during the past few years, initially on the short-term culture of normal peripheral blood lymphocytes and, more recently, on the establishment of such cells in continuous culture, the progressive refinement of in vitro plating, cloning and synchronization techniques and the adaptation of model enrichment and selective situations is viewed as providing a meaningful base for the proposed activity. The impressive array of tools and approaches of microbial genetics should now be broadly applicable to in vitro study of immunocompetent cells. To be truly effective, such programs would involve a multidisciplinary approach combining the skills and ideas of cell biology, cellular immunology, immunochemistry, cell genetics, microbial genetics, cell differentiation and hematology. Predictably emergent would be greatly enhanced knowledge of the magnitude and character of the heterogeneity of the cell population of individual donors, cell kinetics, differentiation, preferential selection or enrichment of sublines and the character of the regulatory processes that govern and control these activities. It is anticipated that the knowledge obtained would be basic and comprehensive in character and quantitatively so massive as to surpass in efficiency the present manner of obtaining information via conventional individual research grants in immunology.

The prospects for additional program projects remains to be determined. However beneficial an effect such projects would have in advancing essential knowledge on the life history of lymphocytes, the fact is that within individual institutions the number and distribution of investigators, with the requisite diverse talents may well prove to be limiting factor. Accordingly, it is likely that even energetic pursuance of these goals will see the emergence of no more than very few such projects. However, the establishment of even a small number of such distinctive program projects could exert a major effect on the direction of and rate of progress in immunology in the United States.

AI BRANCH PROGRAMMING

Activities during this period have been concerned with additional or expanded investigations in the following subjects considered of unusual import for the two special emphasis areas assigned to this Branch and in the planning of conferences that have a special impact on the discipline of immunology:

1. Preferential enhancement of cellular immunity by fractions derived from Mycobacteria.
2. Selective activation in vivo of tumor immunity.
3. Biologic attributes of lymphocytes in established continuous culture.
4. The orientation and organization of the 6th Leucocyte Culture Conference to be held June 1971 was recast with greater emphasis on an expanded interdisciplinary communication in lymphocyte biology.
5. The organization, planning and scientific program for the First International Congress of Immunology was completed.
6. In view of renewed emphasis and expansion of activities in clinical immunology, Academic Press decided to establish a new journal to cover this field and immunopathology.

RESEARCH GRANT HIGHLIGHTS

AI-08579 - S. R. Cooperband

Both humoral antibodies and cell-mediated responses against tumor antigens are generally weak and ineffectual *in vivo*. The possibility of enhancing such reactions by conjugating antibodies with a potent toxin was therefore undertaken. The experimental system utilized tissue cultures of monkey kidney cells infected with mumps virus, guinea pig antibodies directed against mumps virus, and diphtheria toxin. Diphtheria toxin was conjugated to anti-mumps antibody and partially purified. The toxin conjugated antibody was then added to virus-infected tissue cultures and to uninfected control cultures. Mumps-infected cultures displayed extensive disruption within 24 hours of exposure to the toxin-antibody conjugate. Identically treated control cultures, in contrast, showed no ill effects within this time period. Infected cultures treated with unconjugated mumps antiserum also remained intact, demonstrating that antibody alone was insufficient to cause disruption.

To rule out the possibility that the differential effects of the conjugate depend upon greater susceptibility of infected cells to toxic disruption, infected and control cultures were treated with unconjugated toxin. Both populations proved equally susceptible to the free toxin, indicating that selectivity of the conjugate was determined by its antibody component. These results indicate that antibodies conjugated with a cell toxin can indeed act as a "magic bullet," delivering toxic chemicals with great selectivity to specific cell populations.

AI-06918 - H. J. Winn

Remarkable as it may seem in view of the great body of work on H-2 antigens, little if any studies have been directed towards preparation of potent heteroantisera. Water soluble transplantation antigens were prepared from L1210 leukemia cells (DBA/2-H-2d) by the method of sonication and partially purified by ultracentrifugation at 600,000 g and $(NH_4)_2SO_4$ precipitation. Further purification involved starch block and polyacrylamide electrophoresis, and the antigenic activity measured *in vitro* by the inhibition of ^{51}Cr release in a cytotoxic assay and by conventional *in vivo* studies. The soluble H-2 antigen thus obtained was injected (x 3) intravenously into rabbits and the resultant sera tested on mouse spleen cells by a cytotoxic assay. Prior to absorption, the sera reacted with *all* mouse spleen or tumor cells tested. After absorption with selected cell types, antibody activity remained, which was able to detext individual $H\text{-}2^d$ specificities. The strongest response was to H-2.31 with weaker responses to H-2.4 and H-2.13. Thus, in this heterologous system "K end" antigens were more strongly immunogenic than "D end" antigens. The results with water soluble antigens confirm the original finding of Gorer (1936) and the current findings of Sachs (1970), that the rabbit recognizes individual H-2 antigens as well as mouse "species" antigen.

AI-07001 - R. Billingham

When adult (CBxMHA) F1 hamsters are injected with lymphoid cells from MHA donors sensitized to CB antigens, two distinct phases of graft-versus-host disease ensue: (a) an early acute phase consisting chiefly of destructive processes-epidermal necrolysis, bone-marrow aplasia, hemolytic anemia, wasting and death; and (b) a later, chronic phase characterized by immunologic deficiency seen in animals surviving the acute phase. Few, if any, of the acute manifestations are immunologically specific, but rather result secondarily from the primary and only specific immune event: engagement of host and donor lymphoid cells throughout host tissues. Mitotic activity was measured in lymphoid and in non-lymphoid tissues (epidermis and bone-marrow) during the GvH process. It was found that increased proliferation of host lymphoid cells was mirrored by the epidermis (125-IUDR) and erythropoietic cells (59-Fe). Moreover, cessation of replication followed by necrosis was synchronous in all three tissues. From this, it was postulated that the GvH process is pathogenic primarily to somatic cells undergoing replication. Consequently, the higher proportion of dividing cells in skin, marrow, and lymphatic tissues accounts for localization of destruction to these sites. Whether the primary pathogenesis proves to be the donor-host lymphoid cell interaction itself and/or its macromolecular products, the net effect on host tissues bears great similarity to the pharmacologic action of "cycle-active" agents which selectively destroy cells entering the mitotic cycle.

AI-10173 - J. J. Vazquez

Because products released by sensitized lymphocytes are responsible for several *in vitro* effects on macrophages, the influence of these products on phagocytosis was investigated. When lymphocytes from tuberculin-sensitive guinea pigs were cultures in the presence of PPD, the sensitized lymphocyte-culture supernates caused *in vitro* macrophage aggregation and chemotaxis, and significantly inhibited the capillary migration of normal macrophages. In order to properly assess phagocytosis, this aggregating property was overcome by using cultured macrophages which had become adherent to petri dishes. Phagocytosis was determined by measuring the radioactivity of macrophages from sensitized and non-sensitized animals following incubation with opsonized, chromium-51 labeled sheep erythrocytes in the presence of culture supernatants. Culture supernatants stimulated a several-fold increase in the phagocytic activity of macrophages from non-sensitized animals, but the sensitized macrophage population responded less to this stimulatory effect of culture supernatants. The findings suggests that some lymphocyte product may well be responsible for enhanced phagocytic activity of macrophages during the delayed hypersensitivity phenomenon, and the apparent *in vitro* difference in the culture supernatant stimulatory effect on macrophage populations from sensitized animals may be related to prior *in vivo* exposure to lymphocyte products.

K4 AI-46,371 - P. R. Glade

Human lymphoid cells from established suspension cultures migrate *in vitro* like guinea pig peritoneal macrophages and are responsive to MIF, the migration inhibitory factor. Lymphoid cell line supernatants contain a factor which inhibits the migration of guinea pig peritoneal macrophages and cultured lymphoid cells. Lymphoid cells used as indicator cells in the capillary tube migration technique appear to be more sensitive than the widely used guinea pig peritoneal macrophages. Non-lymphoid cell lines, including Hela, fetal lung tissue, and malignant brain tumor tissue from hamsters, were found to contain MIF-like activity in their supernatants. Sephadex column separation of these supernatants indicates that the MIF produced by non-lymphoid cell lines appears in the same peak as that produced by lymphoid cell lines. The fundings indicate that MIF considered to be a soluble mediator of cellular immunity is not solely a lymphoid product but can be produced by any activated cell.

AI-00798 - R. A. Good

Continued investigation of the effects of chronic protein depletion on the immune system of inbred mice has indicated that it enhances considerably cell-mediated immunity, whereas it has minimal and variable effect on humoral immunity. Experiments were carried out on depleted and normal mice to determine whether enhancement of cellular immunity is achieved by altering responsiveness of the effector cells themselves or by some other mechanism. *In vitro* studies of the response of lymphoid cells of depleted animals to PHA and to stimulator cells with major histocompatibility differences indicated an increased responsiveness as compared with cells from normal animals. Since this increase could be due to altered activity of "helper" macrophages, macrophage activity alone was also measured. Peritoneal macrophages from depleted mice ingested significantly more *Listerella monocytogenes* than did macrophages from control mice. The enhancement of cellular immunity might also be due to increased output of thymic "hormone." However, thymectomy did not alter significantly the rejection time of allogeneic skin grafts on nonthymectomized mice on low and high protein diets. Thus, the critical parameters of cellular immunity are affected by protein depletion and the effect does not seem to be exerted at the level of accessory elements of this system.

B. BACTERIOLOGY AND MYCOLOGY BRANCH

The approximate level of support given bacteriology and mycology:

Type of Grant	Number	Amount
Research grants	311	$10,345,000
Training grants	54	2,719,000
Fellowships	49	966,000

Summary

During the year, there has been consistent progress in many of the fields of research supported by the Bacteriology and Mycology Branch of the National Institute of Allergy and Infectious Diseases. All areas have continued at approximately the same satisfactory level of activity without any major shifts in program emphasis being indicated.

Aside from the special emphasis areas of Streptococcal Diseases and Sequelae and of Drug Resistance, research interest continues to be maintained in a number of other fields, for example, in mycotic infections as well as in the pathophysiology and bacterial changes of the flora in the human gut. This interest may be the result of increasing numbers of patients on immunosuppressive therapy who commonly suffer from very serious and often fatal infections caused by opportunistic organisms of relatively low pathogenicity.

Two workshops were organized and supported by the Extramural Programs of NIAID during this year. One was to consolidate and update the latest relevant information relating to Mycoplasmas as agents of disease. It is anticipated that the results of this workshop will be published in one of the widely read journals of medical research. Another workshop on Neisseria biology explored the existing gap areas of basic research knowledge relating to gonococcal venereal disease. A third activity was the partial support by NIAID of a symposium on Streptococci and streptococcal diseases held at the University of Minnesota Medical School.

RESEARCH GRANT HIGHLIGHTS

Some of the more noteworthy accomplishments during this period by grantees in the Bacteriology and Mycology program are briefly summarized here:

Streptococcal Diseases and Sequelae

AI-06308-07 - Vincent T. Andriole

It was demonstrated that hyperestrogen levels, hypertonicity and increased tissue pressure enhanced susceptibility of the kidney to bacterial infections. Increased tissue pressure is associated with a decreased capillary flow and delayed delivery of leukocytes to the area of infection.

AI-09645-02 - E. M. Ayoub

Findings suggest that degradation of streptococcal cells ingested by leukocytes is effected by lysosomal enzymes which lead to digestion and solubilization of various components of the streptococcal cell. Studies with preparations of the enzyme B-N-acetylglucosaminidase from patients during the acute phase of rheumatic valvular heart disease yielded an isozyme pattern different from that of the enzyme from normal leukocytes. Investigation of variations of this enzyme also show some differences in activity of macrophage and leukocyte enzyme preparations which correlate with variations in the isozyme pattern.

AI-08429-05 - R. M. Krause

Dr. Krause has succeeded in obtaining in rabbits streptococcal antibodies with molecular uniformity that can be used for studies on the chemical structure of antibodies and the topography of the antigen binding site. Studies on the immunochemistry of streptococcal mucopeptides underscore the well-recognized fact that there is widespread cross-reactivity among mucopeptides of many bacteria based on a similarity in chemical structure. It is obvious that an understanding of the fundamental nature of the immune response which will come from this discovery is a necessary foundation for research into all diseases with immunological pathogenesis.

AI-06487-07 - D. W. Watson

Dr. Watson has continued his investigation of the streptococcal pyrogenic exotoxins. The A toxin has now been isolated and characterized; these pyrogenic exotoxins are now known to be proteins complexed with hyaluronic acid. There is no evidence that the hyaluronic acid plays any role in toxicity; it acts simply as a carrier substance. The pyrogenic toxin is heat labile, is antigenic and can immunize animals against toxic activity. One possible role of these toxins is in the production of the acute tissue damage necessary to evoke the postulated immune mechanism from cell-associated toxins of Group A streptococci.

AI-09527-06 - L. W. Wannamaker

At a mid-western Indian reservation, studies indicated that streptococci of a specific strain was very common in skin infections (impetigo). For the first time, these skin infection strains were also definitely implicated in cases of acute nephritis here in the United States. In Trinidad, in 1966-1967, a similar epidemic of nephritis caused by this skin pathogenic strain was also implicated. Studies on animals as reservoirs of pyodermal streptococcal strains are in progress, as well as on the importance of local skin factors in the spread of this infection.

Renal Diseases

AI-06964-05 - K. L. Vosti

Studies are continuing on the analysis of factors involved in the pathogenesis of an experimental model of post-streptococcal glomerulonephritis in rats.

A suitable animal model would enable more rapid progress to be made in understanding the development and, hopefully, the prevention and treatment of this disease. A significant accomplishment has been the separation and identification of the glomerular bound antibodies. The implication of these results is that M protein-anti-M protein complexes may mediate the production of this disease. Currently attempts are being made to produce the disease experimentally with the complexes made in vitro. M-reactive fragments have been found to fix to the glomerular basement membrane in a manner similar to that described previously.

T01 AI-00030-11 - J. P. Sanford

Results of research using a rabbit model of pyelonephritis have shown that the kidney responds to bacterial infection with a local immune response of enhanced protein and IgG antibody synthesis. The infected kidneys did not synthesize, to any great extent, any "anti-kidney" antibody; the hypothesis that an autoimmune process contributes to this disease pathogenesis is not well supported. Both normal and infected animals demonstrate a persistent bladder antibacterial effect against a number of different infecting bacterial strains. These organisms were readily killed by contact with bladder mucosa.

AI-04527-08 - L. Accinni

The major objective of this work is the study of the pathogenesis of glomerulonephritis and of vascular lesions in human renal and liver transplants. This group has reported findings that are consistent with the hypothesis that antigen-antibody complexes, formed by denatured DNA specific antibody and complement, may contribute to the pathogenicity of glomerulonephritis. These findings included studies on a mouse and rat model of glomerulonephritis. It was also demonstrated that the original glomerulonephritis present in the recipient may be transmitted to the renal graft, thus indicating an area of serious concern even after perfection of control of the transplant rejection phenomenon.

Staphylococci

AI-07716-04 - R. D. Ekstedt

Results of complex analyses indicated that teichoic acid antigen from all strains of *S. aureus* do not necessarily have N-acetylglucosamine as the immunodominant group, as reported by others. By use of these teichoic acid-antigen complexes, sheet erythrocytes sensitized with this material can now form the basis of a serological assay for teichoic acid serum antibody. Exicting possibilities are now opening for the development of a reproducible serological test for diagnosis of staphylococcal infections.

AI-08906-03 - R.-U. Haque

Selected staphylococcal strains have been tested for production of various toxins and extracellular enzymes. Six different hemolysins, two gelatinases, three lipases, one coagulase, and one lethal factor have all been identified as biologically distinct entities. These are now being characterized and purified, and antisera are being prepared. Except for the various hemolysins, the role of these enzymes and toxic factors in the pathogenesis of staphylococcal disease is still unknown. Pathogenesis of staphylococcal disease is apparently more complex than has previously been recognized, however this study is contributing to better understanding of this pathogenesis.

Venereal Disease

AI-02336-13 - P. H. Hardy

Research on the spirochetal organism *Treponema pallidum* (the agent of syphilis) has as one of its goals the identification of spirochetes within human tissues. Strains of *T. pallidum* from penicillin-treated late and early cases of syphilis have shown that ability of this organism to survive penicillin therapy in the host is not based upon antibiotic resistance. In electron microscope studies, these workers were unable to find any distinguishing features that could differentiate the various pathogenic treponemes from one another.

Drug Resistance

AI-08955-03 - P. D. Hoeprich

In studies with methicillin resistant staphylococcal strains, it was found that the cell protein fraction exhibiting maximal drug binding was not associated with cell walls. There is no clue to the mechanism of methicillin resistance in *Staphylococcus* so far, except that methicillin resistance seems to go along with a reduced capacity to bind the drug. These workers are also studying resistance to rifampin in *Neisseria meningitidis*; they found that large concentrations of this drug rapidly built up in tears and saliva of patients, whereas drugs known to be of no value in chemoprophylaxis of this organism reached only barely detectable levels. The possibility is that *N. meningitidis* may develop resistance to rifampin, since massive, one-step resistance to rifampin has been seen with *S. aureus*. There is no evidence of heterogeneity of susceptibility to rifampin seen so far in cells of *Neisseria* examined.

AI-08362-04 - D. H. Smith

This study on R factors focuses on two separate transferase enzymes considered as the basis of resistance to chloramphenicol and streptomycin respectively. In *E. coli*, it was observed that cyclic adenosine monophosphate affects the synthesis, rather than the activity, of these two transferase enzymes. In *Proteus*, one of these enzymes is synthesized, presumably coded by a structural gene on the bacterial chromosome. Many of the studies designed to unravel the origin of drug resistance will be facilitated by the availability of the R factor which contains structural *loci* for each of six antibacterial drugs. These studies will extend our current knowledge on the origin of R factor genes and the regulation of enzyme synthesis.

AI-07715-04 - S. Cohen

This project is designed to examine the structure of R factors in antibiotic-resistant enteric bacteria and the mechanisms of R factor-mediated resistance. Previously, reports were made on the spontaneous frequent transition of R factor-mediated tetracycline resistance in *Shigella* from one phenotype to another. Recently a gentamycin-resistant strain of *E. coli* from a urinary infection was found to be conjugally transferable, evidently as part of an R factor-mediating resistance to six other unrelated antibiotics. The significance of these observations is that they show R factor-mediated resistance to certain antibiotics may be the resultant of interaction of host cell and R factor-determined properties, rather than a single-step transition from susceptibility to resistance. Clinically significant antibiotic resistance mediated by R factors is therefore a potential threat to antibiotic treatment of enterobacterial infectious diseases.

AI-05941-07 - W. R. McCabe

Efforts have been made to develop an experimental model of an acute staphylococcal endocarditis infection to study the effects of protein binding on therapeutic efficiency of various antibiotics. A successful model was developed by use of a plastic catheter inserted in the animal's left ventricle; use of this catheter created a typical bacterial endocarditis lasting for three to five weeks. By this means, studies are now continuing on equivalent blood levels of various penicillin derivatives to determine a rational therapy. Prevention of staphylococcal carriage in nasal mucosa by means of nasal antibiotic treatment is also under study, because it has been determined that staphylococcal infection rates are greatest among so-called carriers of this microorganism.

AI-09108-03 - W. V. Shaw

Study of the enzymatic mechanism of chloramphenicol resistance in staphylococcal and enteric bacteria has defined precisely the chloramphenicol acetyl transferase involvement in the observed resistance. In *Proteus* species, this enzyme has also been found at a high level in spontaneous and induced chromosomal mutants at a level comparable to the resistant strains. In resistant strains of *S. aureus* the inducible enzyme was found to be identical to that found in clinical isolates of *S. epidermidis*. The resistance in both species is apparently plasmid-linked; there is no evidence for an inter-species genetic interchange between these organisms.

Enteric Microflora Ecology

The competitive and synergistic interaction of microbes in the anaerobic flora of the mammalian gut are of especial interest in microbial ecology.

AI-07328-05 - R. Freter

The objective of this work is to study the mechanisms by which the normal intestinal microflora maintains its stability and prevents growth of pathogens within the gut to establish maximally beneficial species with minimum detriment to the patient.

AI-08254-03 - D. C. Savage

Many anatomic and physiologic characteristics in animals are influenced by microorganisms of the intestinal flora; these influences may be important early in life. The studies by Dr. Savage can help to explain the pathogenesis of bowel diseases of microbial etiology, an area where more knowledge is sorely needed. For example, the metabolism of bile steroids by enteric microflora, it is now believed, may be implicated in cancer of the colon. Results of work reported in this study indicate that in animal models early malnutrition and infectious diseases, especially bowel disease, act synergistically to limit growth and quite possibly intelligence.

Mycology

AI-07000-06 - B. J. Wilson

Sweet potatoes damaged by molds caused numerous cattle deaths in a recent outbreak in Georgia, with pulmonary edema as the most prominant symptom. In other similar cases, a similar toxin was found to cause severe liver damage. These toxins, produced by certain fungi, are of importance not only because of direct loss of livestock, but also because of their great potential involvement in human liver disease in areas of the world where yams and sweet potatoes are a staple of the diet. In another study, a strain of *Penicillium* formed a substance on corn causing focal liver necrosis in cattle. Feedstuffs which harbor large and diverse populations of various fungi could under certain specific conditions of temperature and humidity be directly implicated in toxin deaths in livestock, and most probably in man as well. The carcinogenic mycotoxin, aflatoxin, is a prominant example of this potential threat.

AI-05022-08 - G. S. Bulmer

Seventy-five percent of the peripheral leukocytes from normal humans phagocytize and kill nonencapsulated cells of *Cryptococcus neoformans*. However, cryptococcal capsular material is a specific inhibitor of this process and may therefore function as a virulence factor. Patients with cryptococcus poorly phagocytize this yeast, owing to the presence of the cryptococcal capsular material in their serum. The infectious particle of *C. neoformans* is probably a nonencapsulated yeast of a size less than 4 microns. After entering the human being, about 5-10 hours' incubation is required for the production of enough capsular material to cause a 50% inhibition of phagocytosis. The capsule itself is composed of a number of complex sugars and sugar acids with an estimated molecular weight of 15,000; it appears to adhere to the cell wall at specific receptor sites.

C. BIOCHEMISTRY AND PHYSIOLOGY

The approximate level of support given biochemistry and physiology:

Type of Grant	Number	Amount
Research grants	349	$13,301,000
Training grants	8	350,000
Fellowships	49	610,000

Summary

Antiviral substances continues as the sole special emphasis program in this Branch. Research involving the bacterial viruses (bacteriophages) constitutes a highly interesting and basic aspect of the program. As of January 31, 1971, the Institute had an investment of approximately 3.9 million dollars in bacteriophage research. Another aspect of the antiviral substances program involves interferon. As of January 31, 1971, the Institute had an investment of approximately 1.5 million dollars in interferon research.

RESEARCH GRANT HIGHLIGHTS

AI-08388-04 - D. Baltimore

Dr. Baltimore recently received international recognition for his contribution in the area of reversal of information flow in the growth of RNA tumor viruses. Much excitement was generated when Dr. Baltimore reported that RNA-dependent DNA polymerase activity could be found in the virions (virus particles) of certain RNA tumor viruses. Rapid confirmation of these findings and their extension by many other investigators has left no doubt that RNA-dependent DNA polymerases exist in the virions of all RNA leukemia and sarcoma viruses and also in the mouse mammary-tumor virus.

This extremely important discovery which upsets the Watson-Crick dogma represents a great advance in the area of medical science with important implications for a better understanding of the viral etiology of cancer. Dr. Baltimore recently received the Warren Prize, a triennial award given by the Massachusetts General Hospital, for outstanding research. In addition to this, he was the recipient of the Eli Lilly Award in the areas of microbiology and immunology.

AI-05669-08 - E. Corey

Dr. Elias Corey of Harvard University has announced the laboratory synthesis of the last two of the body's primary prostaglandins, a series of hormone-like substances which may find wide use in birth control and the treatment of asthma, ulcers, and high blood pressure. Apart from the work on synthesis in his laboratory, there are no other existing syntheses which yield the hormones in optically active, naturally occurring form. His process is used in several pharmaceutical houses to prepare prostaglandins for use in medical studies. He understands that these companies are projecting for the immediate future the synthesis of prostaglandins on a scale of several hundred grams per month. He has elected to keep his process unencumbered by patents so that it is freely available for the benefit of mankind.

AI-06182-06 - C. P. Schaffner

Dr. Schaffner has found that the biochemical basis of bacterial resistance to the antibiotic gentamicin in six clinically isolated strains of *Pseudomonas aeruginosa* is a change in the sensitivity of the ribosomes. The present study confirms that the primary action of gentamicin C is localized in the ribosomes. The present results are in accord with the fact that R-factor-mediated gentamicin resistance of high levels has not been observed and the levels of resistance mediated are very low.

AI-03772-11 - W. B. Wood, Jr.

Dr. Wood, whose untimely death in March 1971 cut short a brilliant and productive research career, reported that suppression of the pyrogen-producing capacity of exudate granulocytes results from incubation of the cells in plasma, serum, or Ringer's solution. The suppressive effect is reversible and appears to involve the cellular uptake of calcium ions. In contrast, regeneration of pyrogen-producing capacity in depleted exudate cells occurs only when the cells are incubated in serum.

AI-05629-08 - T. Merigan

Dr. Merigan recently conducted comparative studies of a new, small molecule, orally active interferon inducer, that is Bis-DEAE-Fluorenone, and double stranded RNA which revealed the orally active substance to have a much lower therapeutic index and hence of much less practical significance for potential treatment of human disease. Several *in vivo* infections were studies with synthetic double stranded polynucleotides and interferon. These include intranasal vesicular stomatitis virus, *Mycobacterium leprae*, radiation induced lymphoma virus, Moloney sarcoma virus, and *Listeria monocytogenes*. In all of these settings, except for *Mycobacterium leprae* infection, effects of polynucleotides which are beneficial for the host have been observed. In the intranasal VSV infection, where the most detailed studies have been carried out, it is clear that all the antiviral effects observed relate directly to the amount of interferon stimulated. In tissue culture, two viruses have been studied to find interferon's level of action. Both rubella virus and Sendai virus can be inhibited in their replication in tissue culture with appropriate interferon preparations, and it appears as though in both cases viral RNA synthesis is inhibited in parallel with this antiviral action.

D. PARASITOLOGY AND MEDICAL ENTOMOLOGY

The approximate level of support given parasitology and medical entomology:

Type of Grant	Number	Amount
Research grants	176	$5,913,000
Training grants	25	1,055,000
Fellowships	22	357,000

Summary

Nearly one-third of the research grant budget for the Branch was used to support studies on three diseases of worldwide importance - schistosomiasis, malaria and amebiasis. The first two are considered to be among the diseases which rank highest in importance on a worldwide basis. Schistosomiasis is presently causing prolonged disability in more than 100,000,000 people throughout the world, and eventually causes the death of a large proportion. Treatment is not satisfactory, and the application of control measures is inadequate. The construction of large dams throughout the world to irrigate large acreage to produce more food produces extensive habitats for the snails which transmit this disease. Thus an expansion of the number of people affected by this disease follows. Malaria is still one of the major disease problems for the armed forces, and much of the research being done on this disease in the United States has been supported by funds from the Defense Department. More recently, the Defense Department has narrowed its support to aspects which are immediately concerned with military problems, and many of the projects they formerly supported are now supported by this Institute. Amebiasis has long been an important disease in this country, as well as in the rest of the world. Although treatment for the disease can be successful if started early, there are many unsolved problems. In addition to the classical intestinal amebiasis and amebic liver abscess, a new form of amebic disease has arisen within the last few years. The amebae concerned belong to a different group which were formerly thought to have no relation to human health. Within the last few years, however, it has been shown that numerous cases of meningitis have been the result of acquiring infection with amebae from swimming in ponds. A number of grants have been awarded to support research on this disease entity.

One of the important needs in connection with the parasitic diseases is a practical vaccine for prevention of the disease. In order to encourage research in this area, a special emphasis area, "Immunity to Animal Parasites," was established by the Advisory Council two years ago. Some of the projects involve the diseases mentioned above, but others concern a variety of different diseases. All told, the funds devoted to this special emphasis area represent about one-fourth of the total research grant budget.

Another one-fourth of the research grant budget is devoted to support of a special emphasis area entitled "Biological Regulation of Vectors." This program includes basic science studies of the insects, snails, mites, ticks and other organisms which transmit human disease. The studies included in the special emphasis area are those which show a potential for developing new methods for the biological control of these vectors. It is considered urgent to find such methods because of the dangers which have been pointed out with regard to controlling the disease vectors by the use of DDT and other potent insecticides.

RESEARCH GRANT HIGHLIGHTS

AI-07724 - R. M. Lewert

Philippine field laborers who are repeatedly exposed to schistosome infection show evidence of having developed a strong immunity to superinfection. The results provide encouragement to seek a means of artificial immunization to protect workers from the severe advanced stages of this disease.

AI-08718 - L. H. Miller

Studies of the rheologic properties of malaria organisms have shown an apparent difference in adhesiveness between two species of malaria. The mechanism of adhesion of infected red cells may be related to the causation of the highly fatal cerebral malaria. The effect of drugs on the adhesiveness can be of great importance in saving lives, and therefore the knowledge concerning differences between species is of potential practical value.

AI-05345 - R. B. Crandall

The acquired immunity to Trichinella is complex and seems to involve both a humoral antibody and antigen reactive lymphoid cells. The relative roles must be further evaluated as a step toward possible artificial induction of immunity. These studies are important for knowledge of this species and also serve as a model for other parasites causing human disease.

AI-09166 - E. D. Box

Toxoplasma is an important but somewhat baffling disease often infecting humans and causing serious damage. Knowledge of the life cycle has recently come to light, but many important details are still unknown. Dr. Box has succeeded in cultivating in vitro a related parasite, the isospora of the sparrow. This finding can open the way for study of the parasites occurring naturally in song birds to show if they can be possible reservoirs of infection. These studies serve as a model for the study of related Toxoplasma of humans.

AI-08207 - R. E. Kuntz

Dr. Kuntz and his colleagues have been searching for suitable non-human primates for the study of Schistosoma haematobium, a parasite which causes serious human disease. They have found that in certain monkeys, the infection causes hydronephrosis, involvement of the ureter and of the urinary bladder very similar to the effects of the infection in man. These experiments open the way for a more thorough understanding of this disease and a possible discovery of new means of treatment and control.

AI-05253 - A. B. Weathersby

Some strains of mosquitoes which transmit malaria show a resistance to infection as compared to other strains which are more susceptible. Dr. Weathersby and his colleagues have found that concentration of extracts of these two groups of mosquitoes included in the diets of mosquitoes kept in captivity can change the degree of susceptibility. These results could be the first step in developing a new method of controlling the transmission of malaria.

AI-05910 - P. H. Silverman

Dr. Silverman and his colleagues report that the induction of artificial immunity can be developed in animals to the large round worm which also infects humans. This is the first time that material extracted from cultures of larvae of these worms have been used to induce immunity. This work is an important step toward the eventual production of a vaccine to protect humans against serious human diseases caused by animal parasites.

AI-07770 - J. F. Schacher

Supported by Dr. Schacher's research grant, Dr. Ash and Dr. Riley report that they have been able to establish infection of the filarial worm Brugia pahangi in the jird (mangolian gerbil). The infections were similar to those which have been described for the cat, which is the ordinary host for this worm. Since this worm is closely related to a species which causes serious disease in man, it is important to have a small animal host which can be conveniently bred and kept in the laboratory. Studies on these animals can now proceed to elucidate many of the problems which cannot be studied in humans.

E. VIROLOGY AND RICKETTSIOLOGY

The approximate level of support given virology and rickettsiology:

Type of Grant	Number	Amount
Research grants	150	$7,389,000
Training grants	32	2,017,000
Fellowships	30	608,000

Summary

Viral diseases undoubtedly present the greatest variety of health problems today that need to be overcome before noticeable advancement against infection diseases can be expected. Diagnosis of many of these is very difficult and therapy questionable in most instances. Progress in the two special emphasis areas of the Virology and Rickettsiology Branch, namely the Chronic and Degenerative Diseases and the Infectious Hepatitis Program, was not encouraging this past year, but evidences of advancement and not-too-distant accelerated progress are beginning to show. The work with Australian Antigen (AA) has given a much better and faster test for serum hepatitis and this lends encouragement for attack on the tough infectious hepatitis problem. Influenza investigations are receiving considerable emphasis by NIAID-supported investigators and it is hoped that progress will be made on this problem before another pandemic occurs.

RESEARCH GRANT HIGHLIGHTS

Chronic and Degenerative Diseases

The chronic and degenerative diseases still present the most baffling and time-consuming investigations on viruses. However, the investigators during the past year have reported encouraging results, especially in work with models and those viruses that are necessary for a clearer understanding of a number of these diseases.

AI-06477 - J. B. Henson

The work of this group in their laboratory and similar investigations by others elsewhere suggest that the lesions in Aleutian disease of mink are immunologically mediated. Their studies incriminate the immune processes of the host in the development of clinical disease and lesions in a persistent virus infection. They also point out the generalization that animals (and probably man) do not tolerate to replicating agents. If this generalization continues to be valid, one must explain viral persistence by other means than tolerance. Further elucidation of the mechanisms producing the anemia in Aleutian disease and equine infectious anemia will have important implications in understanding the pathogenesis of human anemias. It is becoming more evident that chronic virus infections play an important role in genesis of human and animal diseases in areas other than neoplasia. One can suspect that an individual animal or man may harbor a virus for years prior to the development of disease. There is little known, however, about mechanisms involved in chronic host-virus relationships and the ways in which these prolonged infections result in tissue and functional alterations and death. The importance of the studies by this group is that they represent a chronic viral infection with multisystems involvement similar to a number of poorly understood human diseases. Studies of the above diseases can serve as a model system to further our understanding of chronic viral infections and the mechanisms of the lesion induction in these conditions.

AI-09023 - D. Walker

The so-called "slow viruses" have been the subject of increasing scientific interest as suspected agents of such chronic diseases as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis. This interest has led to intensive study of known slow viruses and to efforts to establish animal - especially primate - models of slow virus diseases. Walker and co-workers reported that three squirrel monkeys developed a progressive, fatal nervous disease 11 months after inoculation with the agent of transmissible mink encephalopathy (TME). These, with similar and longer term results with other monkeys are encouraging, relative to attempts to alter clinicopathological manifestations by host manipulations. The squirrel monkey thus may assist research of slow virus infections by providing an inexpensive primate model of a disease of this class.

AI-05682 - G. F. Springer

Work with powerful heterophile antibodies against erythrocytes from various animals which arise in patients with infectious mononucleosis has been the subject of concerted recent investigations by Dr. Springer and his group. They have shown that it is likely that these heterophile antibodies are directed against a surface antigen of the agent causing infectious mononucleosis or one resulting from this action, and that the complementary erythrocyte surface receptors are related chemically to this antigen. Additional information and substantiation of these could lead to immunization procedures against infectious mononucleosis. This fits well with research by Dr. W. Henle and co-workers (K06 AI-22,683) where their recent research has presented new information which should lead to better understanding of this infection.

Vaccination

Vaccinations have long been recognized as risky for many persons and cause of anxiety despite the protection afforded.

AI-05577 - D. Horstmann

The investigation group headed by Dr. Dorothy Horstmann has shown post-partum vaccination of rubella-susceptible women to be practical. They suggest that, since their study shows that rubella vaccine administered in the immediate post-partum period is both safe and effective, determination of rubella susceptibility and vaccination post-partum might well be made a routine part of obstetrical care. The chief sources of infection for young mothers are their own children; therefore, vaccination after birth of the first child could be expected to reduce considerably the number of women at risk.

AI-01632 - C. H. Kempe

Dr. Kempe and his group, in work with immunological problems of smallpox vaccinations, with follow-up of satisfactory results obtained previously, has led to the extension of these experiences and there have now been an additional 2,000 primary vaccinations with the attenuated vaccina strain in normal children without untoward effects and with marked modification of clinical reaction similar to those previously found. There have also been an additional 400 children suffering from eczema vaccinated without evidence of eczema vaccinatum. It is now proposed for the eczematous child who cannot receive primary vaccination, and who may be exposed to siblings who have been vaccinated, that this new vaccine be made available through the various state health departments at an early date. Progress of this type with vaccines makes vaccination programs more effective.

AI-07988 - H. Koprowski

Following differential centrifugation or zinc acetate precipitation, it was possible to fractionate rabies-infected tissue culture fluids into components which exhibited different complement-fixing, protective and immunogenic activities. The virion-depleted fraction showed higher complement-fixing activity than the fraction which contained essentially all the virions. The virion-depleted fraction obtained after precipitation of the virus with zinc acetate showed, in addition to complement-fixing activity, good protective activity. Thus, it is possible to produce a safer vaccination material, which contains a minimum quantity of infectious virus, is immunogenic, and induces protection against challenge with virulent virus.

Hepatitis

AI-09516 - M. Prince

An assay for the serum hepatitis antigen (SH/Australia/hepatitis-associated antigen), which can utilize presently available impure reagents and is able to provide results with sufficient rapidity to permit screening of labile blood products, has recently been reported (Prince & Burke, 1970). This procedure, which they termed high voltage immunoelectroosmophoresis (IEOP), is a modification of the counter-electrophoresis method in which antigen is caused to migrate in an electric field through a layer of agarose against antibody migrating in the opposite direction due to endoosmotic flow. Similar methods of screening for serum hepatitis antigen, which utilizes a lower voltage, have recently been described by other workers in the hepatitis field. Because of its rapidity, this technique has essentially eliminated transfusion of blood subsequently found to be antigen positive. When agar gel diffusion had been employed as a screening technique, approximately 1/3 of the blood subsequently found to be antigen positive had already been transfused when this information became available. This rapid test is thus instrumental in preventing a number of serum hepatitis cases that might result from regular blood transfusions.

Antiviral Substances

AI-00261 - A. M. Lerner

Of 13 patients who had Herpesvirus hominis encephalitis in Michigan during a 30-month period, six were seen within 13 months at a single medical center and treated with idoxuridine (IUDR). In four patients cerebrospinal fluids were normal, but the electroencephalogram was abnormal in every patient. Biopsies of the brain in five patients revealed Herpesvirus hominis in two and intranuclear inclusions in one. Therapy was based on a presumptive clinical diagnosis, later confirmed by a striking rise in complement-fixing antibodies. Four of the six treated patients have recovered and resumed all their usual activities. From these studies, idoxuridine appears to be an effective therapy for this often fatal form of encephalitis.

AI-00299 - E. Jawetz

Dr. Jawetz and co-workers in their work with idoxuridine (5 indo-2-deoxyruidine or IUDR) have concluded that, in spite of its being a highly specialized form of antiviral therapy in treatment of herpetic keratitis and its limitations, it remains an effective method for the treatment of a majority of patients with early epithelial herpetic keratitis. Among patients exhibiting resistance to this treatment, only a small minority are infected with virus strains biochemically resistant to IUDR. Hopefully, further work on this will clarify a number of problems related to its usage.

AI-02953 - M. Ho

Individuals with certain or suspected exposure to rabies are generally given prophylactic treatment, since the disease is uniformly fatal once symptoms appear. Prophylactic measures currently available, however, are unpleasant and hazardous, a fact which has led scientists to seek better alternatives. The antiviral substance interferon has seemed, by Dr. Ho and his group of investigators, to offer particular promise as a treatment for rabies. Normally produced by animals in response to viral infection, interferon is an effective broad-spectrum inhibitor of viral reproduction. Since interferon inducers are known to elicit high interferon titers, the investigators sought to protect rabbits against rabies by treatment with the efficient inducer poly I. poly C. Survival rates of the rabbits experimentally infected with rabies were significantly high when compared with controls. These results indicated that poly I. poly C. administered shortly before or after inoculation with rabies virus not only protected rabbits from the immediate infection, but permitted them to develop active immunity. The investigators believe these observations are pertinent to the problem of rabies in man and may suggest a possible new approach to post-exposure treatment of this disease.

Influenza

Interest in influenza research is continuing and it is hoped that investigators will come through with important findings relative to control prior to another pandemic time.

K03 AI-35,225 - C. F. Phillips

A highly purified influenza vaccine, recently introduced, has raised hopes of an effective immunogenic agent free of side effects. Trial vaccinations by Phillips and co-workers resulted in significant rises in antibody titer on those vaccinated on follow-up studies. Contrasted to previously available influenza vaccine, the highly purified vaccine seemed to be more satisfactory for use in children of school age and should be particularly advantageous for use in children who are being immunized because of underlying debilitating disease since it is apparently free from side effects. Further field evaluation of this vaccine, now available, could help in the overall influenza vaccination problems.

Arboviruses

The arboviruses continue to maintain a threat to large segments of our population and continued surveillance and research are needed to keep abreast of problems arising with these viruses.

AI-03028 - W. Reeves

The arthropod-borne encephalitis program in central California has presented interesting problems relating to possible future epidemics. Reeves reports that recent studies of what are generally regarded as epidemic situations (a) resulted in very few Western Encephalitis (WEE) virus and St. Louis (SLE) encephalitis virus isolations; (b) that there were sufficient Culex tarsalis (prime vector of WEE and SLE viruses) populations to initiate and maintain transmission of cycles of WEE and SLE viruses; (c) there were few WEE and SLE virus isolations while the Turlock virus which uses the same avian hosts and insect vectors did not seem affected; (d) for the first time there was noted widespread resistance to a number of the organic phosphorous insecticides used to control this mosquito; (e) autogeny rates (i.e., ability of female mosquitoes to lay eggs without taking a blood meal) appeared to be high in areas of Kern County where flooding reduced or eliminated the availability of vertebrate hosts. The above raised the question of whether resistance to organic phosphorous insecticides is somehow related to autogeny or susceptibility of the Culex tarsalis mosquito to infection with certain arboviruses. Further studies are needed and planned for these questions that can have a bearing on future epidemic situations.

AI-02686 - W. M. Hammon

Hammon reported that an epidemiologic analysis of the 1962 Tampa Bay, Florida, St. Louis encephalitis (SLE) epidemic showed widespread SLE virus infection of mosquitoes and birds in several counties about the Bay, but the highest rates of clinical disease occurred in elderly white residents of one county, most of whom were recent migrants from northern USA. Lowest rates were in non-white and older white residents who were born in or moved to region prior to 1934, a time at which recurrent extensive dengue epidemics had been recognized. The relatively greater amount of dengue virus immunity in the groups, as measured by laboratory tests, affording at least partial protection against clinically recognizable SLE disease, is proposed as the best hypothesis to explain the observed demographic differences. The investigators believe that eradication of dengue fever vectors may be reflected in later years by greater human populations susceptibility to SLE infection and possible epidemics.

Research Grants Program Analysis

Fields	1971 Estimate
Allergy and other immunologic disorders........	$12,542,000
Transplantation immunology...............	(3,386,000)
Bacterial and Mycotic Diseases................	16,401,000
Streptococcal Diseases and Sequelae.......	(984,000)
Drug Resistance.........................	(6,396,000)
Parasitic Diseases and Problem Areas...........	6,273,000
Immunity to Animal Parasites.............	(1,503,000)
Biological Regulation of Vectors..........	(1,510,000)
Viral Diseases and Problem Areas..............	13,025,000
Infectious Hepatitis.....................	(97,000)
Chronic and Degenerative Diseases.........	(1,563,000)
Antiviral Substances.....................	(4,949,000)
Other Research................................	1,023,000
Subtotal.......	$49,264,000
General Research Support......................	5,044,000
Scientific Evaluation.........................	40,000
Categorical Clinical Research Centers..........	1,266,000
International Centers for Medical Research and Training..................	2,263,000
Subtotal.......	$ 8,613,000
Total, Research grants........................	$57,877,000

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COLLABORATIVE RESEARCH PROGRAMS: SUMMARY

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

FY 1971

During FY 1971 there were no significant organization changes within Collaborative Research, and few changes in personnel. Mr. Curtis Tate, Administrative Officer for Transplantation and Immunology Branch left on June 1970 and was replaced by Mr. C. Eugene Harris.

There was a transition during the year in the method of handling the primary review of contracts. In the past, the regular Advisory Committees reviewed all contract proposals including new ones and renewals; during FY 1971 primary reviews were handled by *ad hoc* groups comprised of regular Committee members, and scientists from NIH Intramural laboratories other government agencies and from universities. This has permitted greater flexibility in scheduling reviews and has given the regular committees a broader role in program review and new program generation.

Emphasis in the Infectious Disease Branch has been focused on the recently established programs on antiviral substances, and smallpox and pneumococcal vaccines. New programs were initiated in the development of vaccines against bacterial meningitis.

The Transplantation and Immunology Program is making a concerted effort on the clinical evaluation of tissue typing in organ transplantation and on the use of immunosuppressants in prolongating organ grafts. Studies on soluble antigens for inducing immune tolerance will continue to utilize a mouse model system.

The Research Resources Program has completed the production, testing and processing of reagents to most of the viruses and mycoplasmas of human health research importance. Future emphasis will be on the development and production of reagents and resources for research on hepatitis and for research in allergy and immunology.

A total of $11,647,000 was apportioned for Collaborative Research for FY 1971, of which $1,946,000 was for the Geographic Medicine Branch. The $9,701,000 apportioned to Collaborative Research, excluding Geographic Medicine Branch was allocated as shown below:

Contracts:

Infectious Disease Branch:

Antiviral Substances Program	$1,075,846
All other IDB	3,496,927

Transplantation and Immunology Branch	1,969,716
Research Resources Branch	1,527,781
Other contracts and agreements	465,730
Personnel, Materials, Equipment, Travel, etc.	761,000
Reserves placed by NIH & NIAID	404,000
	$9,701,000

There were 116 research contracts funded in Collaborative Research totaling $7,801,577. Distribution of contracts by categories are as follows:

	Number	Amount
Profit Making Organizations	17	$1,763,117
Interagency Agreements	7	739,000
Universities	68	4,049,339
Non-profit Foundations	24	1,250,121
	116	$7,801,577

RESEARCH RESOURCES BRANCH

1. Summary

The Research Resources Branch provides highly characterized seed and corresponding antiserum for a variety of viruses and mycoplasma for health research purposes. The program also provides resources for research requiring biophysical instrumentation and particularly zonal centrifuge systems.

The production phase of four major reagent programs is essentially complete. The enterovirus reagent production will be completed with the processing of the antiserum pools for rapid virus typing and the remake of enterovirus reagents which did not meet specifications. Adenovirus reagents of low titer or lacking in specificity are being made using higher standards for production and testing than called for in the original reagents. Two new adenovirus prototypes will be considered for reagent production. Production of seed and antisera to myxovirus reagents is complete. Prospective new reagents include antisera to influenza virus neuraminadase and hemagglutinin. Mycoplasma reagent production has been completed and certification of reagents should be completed in the near future.

The arbovirus reagent program is progressing satisfactorily and bulk and final vial are continually available for systematic evaluation by the certification laboratory. Four arbovirus production contracts have been terminated and two more will be terminated at expiration since program goals should be realized in a year or two. The four dengue arboviruses have not been assigned to a contractor since suitable immunization schedules for the production of specific reagents have not been developed. Three mosquito blood meal antisera have been produced and are currently being tested by the certification laboratory. This will complete the program goal of producing 12 mosquito blood meal antisera.

The project to produce interim reagents for Australia (hepatitis-associated) Antigen was successfully completed in July 1970 through the combined efforts of Intramural scientists, the NIAID/AEC MAN Laboratory and the CDC. These reagents have been highly utilized and program plans call for production of new reagents representing antigenic subtypes. The program has sponsored working conferences designed to obtain advice on projects for reagents and resources for hepatitis research.

Antigen E prepared from dwarf ragweed pollen is being packaged and will be available for distribution in the near future. The cytomegalovirus reagents produced in pilot lots are currently being tested.

The rhinoviruses reagent program for the Phase I rhinoviruses (1-55) is nearing completion with many certified seed virus reagents presently available for distribution. It is anticipated that production of Phase II rhinovirus types (56-89) will commence in June of 1971 and these reagents should be available by late 1972.

With the publication and distribution of the 1970 Research Reagents Catalog there has been a continuing publicizing of the availability of new reagents and reference to the 1970 Catalog is reaching investigators not previously aware of reagent availability.

The program derives continued support of the NIH Intramural Laboratories which furnish scientific and technical consultation, project officers, and ad hoc Committee members for review of new and ongoing programs.

2. Program Status Report

Adenoviruses

Reagents have been prepared for all thirty-one adenovirus types and all are currently being distributed. However, surveillance testing indicates a drop in titer of some adenovirus seed reagents to a level of potency which limits their usefulness as research reagents. Therefore, the following adenovirus seeds are to be remade; 8, 9, 13, 15, 16, 17, 18, 22, 23, 24, 26, and 27; the following adenovirus antisera will be remade due to their initial low titer and subsequent classification as borderline interim reagents; 2, 4, 8, 9, 10, 13 and 14. These remakes should be available the latter part of 1972. There are two new candidate prototype adenoviruses which will be considered for production at the next Research Resources Advisory meeting.

Arboviruses

Four contractors are actively engaged in the production of reference reagents to eighteen arboviruses. The certification laboratory has tested seventy-five arbovirus reference reagents which were subsequently recommended for distribution by the Arboviruses Committee. Thirty-nine bulk reagents are in the process of being tested prior to final processing.

A contract for the production of antibody-containing grouping ascitic fluid has resulted in the production and approval for distribution of eleven grouping fluids. Fourteen others are in various stages of testing prior to final processing. Five are currently in production.

Three mosquito blood meal antisera contracted for have been produced and are currently being evaluated. The addition of these three will make a total of twelve antisera available for distribution. Three for major animal groups and nine for common vertebrate hosts.

Table I summarizes production status of arbovirus reagents.

Myxoviruses

In the initial phase of the myxovirus program reagents were produced to subgroup I and subgroup II viruses and other related virus types. To date there are available for distribution seed and corresponding antisera prepared in roosters to eleven influenza A human and animal strains, five influenza type B strains and one type C strain. It has been the policy of the program to prepare reference reagents to those influenza virus types which are to be included in vaccine preparations.

The following new reagents are currently being tested by the certification laboratory; seed for parainfluenza 4B and antisera to parainfluenza 3 (SF-4), parainfluenza 4B and respiratory syncytial (18537). These reagents should be available for distribution in the near future. The following new reference reagents have been produced and approved for distribution; seed for parainfluenza 3 (SF-4), respiratory syncytial (18537) and parainfluenza 3 (HA-1); antisera for parainfluenza 4A (M-25) and measles. It is anticipated that reagent antisera for neuraminadase and hemagglutinin representing key influenza virus strains will be produced in fiscal year 72.

Enteroviruses

In addition to the reference antisera prepared in rhesus monkeys, sizable volumes of antisera have been prepared in horses. To make the most effective use of these equine antisera; eight grouping pools (Lim Benyesh-Melnick) have been prepared; these pools are now being packaged and will be available for distribution by May 1971. Through the use of these pools, it should be easier and faster for laboratories to type enterovirus isolates.

Rhinoviruses

Phase I of the rhinovirus reagents program to prepare seed virus and guinea pig antisera to fifty-six prototype rhinoviruses is nearing completion. At this time there are forty-five final vial seed viruses that have been approved for distribution as reference reagents; five have been rejected and six are currently being tested. Twenty-two final vial guinea pig antisera are currently being tested, thirty-two bulk antisera are being tested and two bulk antisera have been rejected because of low titer.

Sufficient guinea pig antisera and certified seed is being prepared by the contributors involved in Phase II of the characterization program so that adequate authenticating serum and seed will be available when production of Phase II reference reagents is initiated. Five contracts are currently being negotiated for the production of thirty-four Phase II rhinoviruses and any remakes of the unacceptable Phase I reagents.

The number of accepted rhinovirus prototypes, representing Phase I and Phase II now totals eighty-nine.

Other Viruses

Seed virus and antisera are available for three reovirus types. Among the herpes viruses the program is distributing reagents for herpes simplex, monkey B and pseudorabies.

Cytomegalovirus reagents have been produced and are currently being tested by the certification laboratory.

Interim reagents are being distributed through this program to twenty-six simian virus types.

Mycoplasma

The production of mycoplasma reagents is now completed. This has been a closely coordinated program between the Research Resources Branch, NIAID and the Division of Biologics Standards for the preparation of reference reagents (seed, corresponding mule or donkey antisera) and conjugated serum to twenty-eight mycoplasma species of human and non-human origin.

Seed and conjugated antisera to all twenty-eight types are now available for distribution. Equine antisera are available to nine types; while the other 19 types are either being packaged for distribution or are undergoing miniature proportionate pool testing. Ten of the conjugated sera were made available under an agreement with National Cancer Institute.

Twenty-five seed reagents, seven final ampouled antisera, twenty-one minipools and twenty-eight conjugated antisera have been tested by the certification laboratory. The data obtained are comparable with the results obtained by the producer and the Laboratory of Infectious Diseases.

AEC-NIAID-Molecular Anatomy Program (MAN)

This Interagency Program is serving to develop new separation systems for virus and antibody purification, concentration and analysis with the Molecular Anatomy Program NIAID located in Rockville, near the Bethesda campus. The Principal Investigator, Dr. John Gerin, has been working closely with Intramural scientists on projects involving the study of the respiratory syncytial virus and its temperature sensitive mutants and biochemical studies of mouse hepatitis A-59 strain and considered to be the coronavirus prototype. There has been increasing activity to concentrate, purify and characterize Australia antigen. The satellite facility is now fully operational.

Antigen E

A contractor is currently packaging purified antigen E fractionated from bulk ragweed pollen purchased by the Branch. It is anticipated that the packaged reagents will be available for distribution in the very near future.

Hepatitis

Production of reagents (antigen and guinea pig antiserum) for Australia (Hepatitis-Associated) Antigen (Au) has been completed and numerous laboratories have requested and received these valuable materials since August. Ten WHO laboratories are now participating in a collaborative study to evaluate the Au reagents. This cooperative testing will increase the value of the reagents through multiple independent testing using a variety of serologic tests.

Extensive testing of the originally prepared Au antiserum revealed antibody to human serum components. In order to produce a reagent free of antibody to human serum components, an additional amount of serum was adsorbed by affinity chromatography. The adsorbed antiserum is also undergoing evaluation by the same 10 WHO laboratories and the adsorbed material will be proposed as a WHO International Reference Standard.

Two working conferences have been sponsored. The first, Working Conference on Antigenic Subtypes of Australia Antigen was held January 8, 1971. It was concluded that 1) reagent antisera should be prepared in animals using antigenically diverse sources of Australia antigen as the immunizing antigen and 2) a long range program in the study of antigenic diversity is warranted in view of the national needs for sensitive reagents in screening donor blood.

The second conference held on April 6, 1971 was a Working Conference on the use of Detroit-6 cells for the isolation of Agents from Patients with Infectious Hepatitis. This conference concluded that the suspect agents being carried in Detroit-6 cells were parvoviruses closely related to the Kilham rat virus.

A contract is now being negotiated with the Delta Regional Primate Center to determine the responses of Australia antigen negative chimpanzees to the Australia antigen and other antigens provided by NIH.

Distribution of Reagents

The following table summarizes distribution statistics from fiscal year 1964 to date:

Fiscal Year	Period of Operation	Total Transactions	Total Amps and vials Distributed	Bulk
1964	7/1/63-6/30/64	52	2,099	
1965	7/1/64-6/30/65	159	15,168*	
1966	7/1/65-3/30/66	215	15,908*	
1967	7/1/66-6/30/67	269	8,615	1,200 ml
1968	7/1/67-6/30/68	391	31,776	26,224 ml
1969	7/1/68-6/30/69	402	15,805	1,172 ml
1970	7/1/69-6/30/70	474	26,242	11,112 ml
1971	7/1/70-3/31/71 (9 mos)	500	10,661	25,070 ml
	Total	2,462	126,274	64,778

*Approximately half of this number was used for reagent testing and evaluation.

The combined number of transactions to date plus those projected to June 30, 1971 is an increase over last year. This increase can be attributed to the fact that the scientific community is becoming more aware of the availability of standardized reagents through distribution of the catalog and announcements appearing in scientific publications.

Included in the above tabulation are 135 shipments of Australia (Hepatitis-Associated) antigen reagents which became available for distribution in August 1970. Since the announcement was made of their availability numerous requests have been received and most have been honored.

The 1970 Catalog of Research Reagents was completed in November 1970. The Catalog has been sent to approximately 1300 investigators. The Catalog listed and described over 100 new reagents including mycoplasma reagents which had not appeared in the 1968 Catalog.

As to distribution of reagents outside the USA, there have been requests from thirty-four foreign countries this year and reagents have been sent to fifty three foreign countries since the program was initiated.

The present policy for distributing reagents provide guidelines designed to safeguard valuable research resources while at the same time permitting their orderly distribution to the research community. There will be continued emphasis placed on publicizing the availability of reagents and their characteristics and at the same time developing effective means for retrieval of test data from program users.

Table I. Arbovirus Production and Testing of Arbovirus Reagents to Date

Approved for Distribution		Final Ampoule Being Tested	Bulk Reagent Being Tested
Seed Virus Reagent			
Anopheles A	Mayaro	Cowbone Ridge	West Nile
Bimiti	MML	Punta Toro	Main Drain
Buttonwillow	Modoc	Melao	Sindbis
Bwamba	Naples SFF	SLE	Sawgrass
Calif Enceph	Nepuyo	Powassan	JBE
Catu	Oriboca	VEE	Tembusu
Chagres	Oropouche	Tacaribe	Junin
Changuinola	Patois		Congo
Colorado TF	Rio Bravo		Cocal
EEE	Sicilian SFF		
EHD	Silverwater		
Guama	Tensaw		
Guaroa	VSV (Ind)		
Hart Park	VSV (NJ)		
Hughes	WEE		
Ilheus	Wyeomyia		
Kern Canyon	YF (17D)		
Manzanilla	Zegla		

Approved for Distribution		Final Ampoule Being Tested	Bulk Reagent Being Tested	
Immune Ascitic Fluid				
Anopheles A	Mayaro	Guaroa	Sawgrass	Sindbis
Buttonwillow	Melao	EHD (NJ)	SLE	Nepuyo
Bwamba	Modoc	Patois	YF (17D)	Bimiti
Calif Enceph	Naples	Cowbone Ridge	Rio Bravo	
Chagres	Oropouche	Hart Park	Wyeomyia	
Colorado TF	Sicilian SFF	Turlock	Tacaribe	
Guama	VSV (Ind)	Hughes	Junin	
Ilheus	VSV (NJ)	Silverwater	Congo	
Group A	Group Guama	MML	Cocal	
Group B	Group Simbu	Manzanilla	Kern Canyon	
Group C	Group VSV	EEE	Tensaw	
Group Capim			Silverwater	
Group Tacaribe			Oriboca	
Group Bunyamwera			West Nile	
Group Quaranfil			Main Drain	
			WEE	

3. Contract Narratives

Arbovirus Reagent Program

AMERICAN TYPE CULTURE COLLECTION (PH43-68-1008)

Title: Catalogue of Arthropod-borne Viruses of the World

Project Director: Dr. Trygve O. Berge

Project Officers: Dr. Earl Chamberlayne and Mr. Robert M. Pennington

Objectives: To maintain a registry of data derived from investigators on arthropod-borne viruses by cataloguing the known physical, chemical and antigenic characteristics, the host and vector range, pathogenesis and geographical distribution of these reagents.

Major Findings: To date nine new catalogue sets have been issued and the active list of participants included 148 individuals or organizations. Distribution of the Catalogue materials involved 59 mailings within the continental USA and 89 to foreign addresses in 48 countries. Distribution by continent was as follows: Africa 12; Australia (including Hawaii) 8; Europe 28; North American 64; and South American 16.

Significance to NIAID Program and Bio-Medical Research: The continued dissemination of the scientific information pertaining to arboviruses is a dynamic contribution to research in arbovirology.

Proposed Course of Project: Since the Project Director is leaving ATCC, it is anticipated that this contract will be continued at another location in FY 1972.

Date Contract Initiated: June 1968

Current Annual Level: $28,370

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (PH43-68-679)

Title: Production and Testing of Arbovirus Research Reference Reagents

Project Director: Dr. Edwin H. Lennette

Project Officers: Dr. Karl M. Johnson and Mr. Robert M. Pennington

Objectives: To produce research reference reagents to St. Louis Encephalitis, Rio Bravo, Yellow Fever (17D), Tembusu, WN, Bussuquara, RSSE, JBE, Junin and one other to be assigned.

Major Findings: In the past year production and testing have been completed on SLE, Rio Bravo, YF, Tembusu and WN. Production has recently been initiated on RSSE, JBE and Junin.

Significance to NIAID Program and Bio-Medical Research: This contract will furnish ten arbovirus reference reagents to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients or arbovirus reagents.

Proposed Course of Project: Subject to performance review, it is anticipated that this project will be renewed for an additional year as part of a continuing arbovirus reagent production effort.

Date Contract Initiated: April 22, 1968

Current Annual Level: $46,225

CENTER FOR DISEASE CONTROL

Title: Production and Testing of Arbovirus Research Reference Reagents

Project Director: Dr. Morris T. Suggs

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: To produce and test Hughes, Silverwater, WEE, VEE, Cowbone Ridge, Tamiami, Sawgrass, Microtus, Bluetongue and Trivittatus.

Major Findings: During the past year certification testing of Cowbone Ridge, VEE, WEE, Tamiami, Sawgrass and Microtus was initiated.

Bwamba, Colorado Tick Fever, Hughes and Silverwater had been approved for distribution.

Significance to NIAID Program and Bio-Medical Research: This project will make available eleven arbovirus reference reagents to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients of arbovirus reagents.

Proposed Course of Project: Subject to performance review, it is anticipated that this project will be renewed for an additional year as part of a continuing arbovirus reagent production effort.

Date Contract Initiated: May 1, 1966

Current Annual Level: $45,000

YALE ARBOVIRUS RESEARCH UNIT (PH43-66-460)

Title: Testing and Identification of Arbovirus Reagents

Project Director: Dr. Wilbur Downs

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: To furnish essential testing and certification services for other arbovirus production contractors.

Major Findings: To date these testing services have been utilized to furnish NIAID Staff and Arbovirus Advisory committee with information needed to determine acceptability for distribution of seventy-four arbovirus reagents. Also, serological testing necessary for approval of the next step in processing of bulk reagents has been completed or initiated for thirty-nine arbovirus reagents. In the past year fifty-eight reagents were tested and approved for distribution or processing.

Significance to NIAID Program and Bio-Medical Research: This service is an essential element of the arbovirus reagent program and it is anticipated that its full usefulness is now being realized.

Proposed Course of Project: Continuation during arbovirus production and processing efforts.

Date Contract Initiated: January 19, 1966

Current Annual Level: $86,360

YALE ARBOVIRUS RESEARCH UNIT (PH43-66-551)

Title: Production of Arbovirus Grouping Ascitic Fluid

Project Director: Dr. Wilbur Downs

Project Officers: Mr. Robert M. Pennington and Dr. Robert J. Byrne

Objectives: To produce thirty arbovirus grouping ascitic fluids usable in a CF test and of such a specificity to differentiate one group from another. To furnish thirty inactivated antigens for use as positive controls and one grouping fluid for the detection of adventitious viruses.

Major Findings: The production of grouping ascitic fluid was delayed for about a year because of an ectromelia virus infection of the mouse colony and the discovery of a mouse brain tissue antibody in some of the reagents. The following grouping fluids have been produced and approved for distribution; Group C, Group Guama, Group VSV, Group Tacaribe, Group A, Group Bunyamwera, Group Simbu, Group B, Group Capim and Group Quaranfil. Fourteen grouping fluids are currently in the bulk testing phase and five are currently in production. Ten inactivated CF antigens are being packaged and twenty others are in various stages of production.

Significance to NIAID Program and Bio-Medical Research: These grouping reagents are an essential part of the total arbovirus reagent program and are designed to make more effective use of the immune ascitic fluid prepared for specific viruses.

Proposed Course of Project: It is anticipated that this contract will be terminated in FY 72 with no additional funding. This will complete the production and testing of thirty-one grouping fluids and thirty inactivated CF arbovirus antigens.

Date Contract Initiated: June 1, 1966

Current Annual Level: No FY 71 funds

LEDERLE LABORATORIES (NIH-69-2170)

Title: Production and Testing of Arbovirus Research Reference Reagents

Project Director: Dr. Paul Vasington

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: To produce arbovirus reference reagents to Modoc, Hart Park, Manzanilla, Montana Myotis Leucoencephalitis, Main Drain, Ross River, Sindbis, Chikungunya, Getah, Bebaru, Hyland J and Lone Star.

Major Findings: During the past year Bebaru, Hyland J and Lone Star were produced and are currently being tested by the certification laboratory. Getah is currently in production. Modoc, Hart Park, Manzanilla, MML, Main Drain, Ross River, Sindbis and Chikungunya had been previously produced by the contractor and approved for distribution or final packaging.

Significance to NIAID Program and Bio-Medical Research: This contract will make available twelve arbovirus reference reagents to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients of arbovirus reagents.

Proposed Course of Project: To be terminated September 1971

Date Contract Initiated: June 1968

Current Annual Level: $17,700 (3 months extension)

UNIVERSITY OF CALIFORNIA (PH43-68-1253)

Title: Testing and Certification of Three Mosquito Blood Meal Antisera

Project Director: Dr. Constantine Tempelis

Project Officers: Mr. Robert M. Pennington and Mrs. Sylvia Cunningham

Objectives: To test and certify three mosquito blood meal precipitin antisera as to titer and specificity during production and following final processing.

Major Findings: These testing services are being utilized to furnish NIAID Staff and Arbovirus Advisory Committee with information needed to determine acceptability for distribution of three mosquito blood meal antisera. Nine mosquito blood meal antisera have been approved for distribution.

Significance to NIAID Program and Bio-Medical Research: The characterized mosquito blood meal antisera will be a valuable research tool in epidemiological studies of diseases transmitted by arthropods by making it possible to identify the blood meal of mosquitoes, phlebotomus, etc., important in the transmission of arboviruses.

Proposed Course of Project: Terminated March 1971

Date Contract Initiated: August 1968

Current Annual Level: $898.00 (one month extension)

UNIVERSITY OF CALIFORNIA (NIH-70-2183)

Title: To Evaluate Methods for the Production of Specific Dengue Immune Reagents

Project Director: Dr. Nyven J. Marchette

Project Officers: Dr. Leon Rosen and Mr. Robert M. Pennington

Objectives: To carry out specific immunization schedules to produce and test eight lots of dengue immune ascitic fluid using six dengue viruses.

Major Findings: The reagents produced are currently being evaluated.

Significance to NIAID Program and Bio-Medical Research: The contractor has attempted to produce highly specific immune reagents which are currently being evaluated. It is hoped that an immunization schedule will result that may be used by other contractors to produce research reagents which are specific for the four types of dengue viruses.

Proposed Course of Project: Terminated December 1970.

Date Contract Initiated: June 1970

Current Annual Level: No FY 71 money

UNIVERSITY OF PITTSBURGH (NIH-70-2184)

Title: To Evaluate Methods for the Production of Specific Dengue Immune Reagents

Project Director: Gladys Sather

Project Officers: Dr. Leon Rosen and Mr. Robert M. Pennington

Objectives: To carry out specific immunization schedules to produce and test eight lots of dengue immune ascitic fluid using six dengue viruses.

Major Findings: The reagents produced are currently being evaluated.

Significance to NIAID Program and Bio-Medical Research: The contractor has attempted to produce highly specific immune reagents which are currently being evaluated. It is hoped that an immunization schedule will result that may be used by other contractors to produce research reagents which are specific for the four types of dengue viruses.

Proposed Course of Project: Terminated September 1970

Date Contract Initiated: June 1970

Current Annual Level: No FY 71 money

UNIVERSITY OF WEST INDIES (PH43-67-65)

Title: Production and Testing of Arbovirus Reference Reagents

Project Director: Dr. Pierre Ardoin

Project Officers: Dr. Karl M. Johnson and Mr. Robert M. Pennington

Objectives: The reagents assigned to the University for production are those arboviruses indigenous to the area or originally isolated in Trinidad and considered to be a potential health hazard to the U.S. mainland.

To produce and test reference reagents for Cropouche, Mayaro, Ilheus, Melao, Guama, Oriboca, Caraparu, Wyeomyia, Nepuyo, Bimiti, Cocal, Catu, Tacaribe, Gumbo Limbo, Lokern, Bunyamwera and Piry.

Major Findings: The following reagents have been produced and certified for distribution: Oropouche, Mayaro, Ilheus, Melao, Guama, Nepuyo, Bimiti, Wyeomyia, Oriboca and Catu.

In the past year Oriboca, Cocal, Tacaribe, and Caraparu have been produced and are currently being tested by the certification laboratory. Production of Gumbo Limbo, Lokern, Bunyamwera and Piry was recently initiated.

Signficance to NIAID Program and Bio-Medical Research: This project will furnish seventeen arbovirus reference reagents to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients of arbovirus reagents.

Proposed Course of Project: To be terminated February 1972

Date Contract Initiated: February 1967

Current Annual Level: $46,680

Adenovirus Reagent Program

FLOW LABORATORIES

Title: Production of Adenovirus Reagents

Project Director: Dr. Jerome Kern

Project Officers: Dr. Malcolm Hoggan and Mr. Robert M. Pennington

Objectives: To produce, test and package AAV free seed virus reagents to 12 adenovirus types and antisera to 7 adenovirus types.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the rhinovirus and myxovirus group, with cold like respiratory diseases.

Proposed Course of Project: To be terminated June 1972

Date Contract Initiated: June 1971

Current Annual Level: $51,702

ABBOTT LABORATORIES (NIH-69-2052)

Title: Production of Reference Reagents to Adenoviruses

Project Director: Mr. George S. Marquis

Project Officers: Dr. Malcolm D. Hoggan and Mr. Robert M. Pennington

Objectives: To prepare purified adenovirus seed free of AAV to types 6, 10, 20, 21, 25, 28 and 29 and antisera to types 20 and 21.

Major Findings: The contractor has had all reagents tested and approved for distribution.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the rhinovirus and myxovirus groups with cold like respiratory diseases. The reagents produced are high titered, specific, AAV free research reference reagents.

Proposed Course of Project: Terminated February 1971

Date Contract Initiated: April 26, 1969

Current Annual Level: No FY 71 money

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (NIH-69-89)

Title: Certification Testing of Enterovirus Reference Reagents

Project Director: Dr. Edwin H. Lennette

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: To perform certification testing of seed virus and antiserum prepared to prototype enteroviruses. In addition, testing to other virus groups will be performed on request.

Major Findings: The certification laboratory has confirmed the test data as given by the producer or in some cases there have been indications of loss of titer and in several cases contamination with other viruses have been determined. The Contractor has completed the certification testing of both seed and monkey antisera prepared for enteroviruses and has completed the testing of the enterovirus pools. Final ampoules of the pools will be tested when available. The contractor has also tested reovirus reagents and is currently testing cytomegalovirus reagents.

Significance to NIAID Program and Bio-Medical Research: The contractor provides data confirming authentication, identification, potency and purity of enterovirus reference reagents, produced by independent contractors. This comprehensive certification testing enhances the value of the reagents as reference standards.

Proposed Course of Project: As long as a contract is in effect for the preparation of enterovirus reference reagents the services of this laboratory will be needed. Since the Baylor University enterovirus contract has been phased out the CSDPH and Center for Disease Control will probably handle future enterovirus program activities for the RRB.

Date Contract Initiated: December 11, 1961

Current Annual Level: $27,437

BAYLOR UNIVERSITY SCHOOL OF MEDICINE (PH43-68-1044)

Title: Enterovirus Antiserum of Equine Origin

Project Director: Dr. Joseph L. Melnick

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: Immunizing antigens are prepared employing the magnesium aluminum carbonate concentrate previously described by Dr. Melnick. Immunizing schedules are studied to determine optimum means for producing high titered serum.

Major Findings: The Contractor has completed the testing of reagents for approximately sixty enterovirus prototypes which were prepared in horses. Immunizing antigens were prepared for use by another contractor. Through a cooperative effort with the WHO Enterovirus Laboratories heterologous testing has been performed and it appears that most of the antisera of equine origin is both potent and specific.

Significance to NIAID Program and Bio-Medical Research: These enterovirus reagents comprise an essential part of a total effort to produce uniformity of virological research results in different laboratories throughout the world. The horse serum which has been used to prepare typing pools is presently being processed.

Proposed Course of Project: To be terminated June 1971

Date Contract Initiated: May 1968

Current Annual Level: $7,905 (3 months extension)

TULANE UNIVERSITY, DELTA REGIONAL PRIMATE RESEARCH CENTER

Title: Studies of response of sero-negative chimpanzees following exposure to Australia Antigen

Project Director: Dr. Arthur J. Riopelle

Project Officers: Dr. Robert Purcell and Mrs. Sylvia Cunningham

Objectives: To furnish healthy sero-negative chimpanzees and to house, feed and provide veterinary care of chimpanzees during pre and post-exposure phases of the study. Contractor will obtain blood samples, biopsy specimens, make clinical observations, and furnish laboratory services.

Major Findings: None

Significance to NIAID Program and Bio-Medical Research: Recent studies of antibody to hepatitis-associated antigen have revealed that a high proportion of normal adults have such antibody. Furthermore, a study of large chimpanzee colonies revealed that most chimpanzees like man are susceptible to HAA positive hepatitis. These studies should lead to information on the response to chimpanzees to infection as measured by serological response and isolation of the organism. These studies form an important base for what must ultimately be obtained, i.e., an animal model system for evaluating vaccines.

Proposed Course of Project: Depends upon results obtained

Date Contract Initiated: June 1971

Current Annual Level: $53,841 (18 months)

BAYLOR UNIVERSITY (PH43-68-1246)

Title: To Conduct a Research Study Leading to the Preparation of Reference Reagents to the Cytomegaloviruses

Project Director: Dr. Joseph L. Melnick

Project Officers: Dr. Julius A. Kasel and Mr. Robert M. Pennington

Objectives: To investigate the propagation of cytomegaloviruses in human embryonic fibroblasts for immunization of primates with infectious cytomegaloviruses to produce a specific and highly potent antisera to each of three strains of cytomegaloviruses.

Major Findings: With all three strains of virus maximum antibody levels were attained after the 5th inoculation, with very little change upon further immunization. The monkey virus strains elicited complete independent neutralizing antibody. Both human strains elicited complement requiring neutralizing (CRN) antibody. The CRN antibody to the human virus was 7S in nature and found to be specific for the immunizing viruses. The best procedure for concentrating virus was a combination of ultrafiltration through Diaflo membranes and subsequent pelleting into a double sucrose cushion. The reagents are currently being tested by the certification laboratory prior to final packaging.

Significance to NIAID Program and Bio-Medical Research: Reagents to the herpes virus group are essential in bio-medical research due to the groups' significant role in human diseases especially of the immunologically-disadvantaged. These viruses all occur frequently as contaminants in tissue culture cell lines, media and biological products.

Proposed Course of Project: Terminated December 1970

Date Contract Initiated: June 1968

Current Annual Level: $15,000 (3 months)

UNIVERSITY OF AARHUS (PH43-67-682)

Title: To Authenticate and Certify Mycoplasma Reference Reagents Prepared to Human and Other Mycoplasma Strains

Project Director: Dr. E. A. Freundt

Project Officers: Dr. Joseph G. Tully and Mrs. Sylvia Cunningham

Objectives: To certify mycoplasma reference reagents prepared to mycoplasma species for identity, potency and purity. Purity testing will be performed with a known spectrum of mycoplasma species including those which are not available in the United States because of importation regulations. In addition, the Contractor will develop new techniques and/or modify or evaluate existing techniques for the detection, identification or characterization of mycoplasma species.

Major Findings: The contractor has tested 1) 21 mycoplasma miniature serum proportionate pools (minipools) and 2 final packaged antisera for homologous titers by DI and IHA, 2) three final packaged antisera against "exotic" strains retested by modified DI technique, 3) 28 conjugated antisera tested for homologous titers and for cross-reactivity with 3 "exotic" strains, by direct immunofluorescence on agar; 4) 45 seed reagents for viable counts; 5) 25 seeds tested biochemically and serologically against homologous and heterologous antisera; and 6) five final antisera (against human strains) tested for homologous and heterologous titers by DI, MI, CF and IHA tests.

In addition, the University of Aarhus has produced small batches of mycoplasma seed and antiserum reagents (against some 40 strains) as required to perform the above testings.

Production, sterility and safety testing of the equine antisera to three exotic mycoplasma species was completed. These antisera have now been tested at Plum Island and are now available for distribution.

Significance to NIAID Program and Bio-Medical Research: Certification of mycoplasma reagents by the University of Aarhus is essential as a safeguard in insuring authenticity and purity. Specialized testing by the University of Aarhus, for which this laboratory is uniquely qualified, will further enhance the value of the reagents as reference standards.

Proposed Course of Project: It is anticipated that this contract will be completed sometime within the next year.

Date Contract Initiated: April 28, 1967

Current Annual Level: $16,052 (6 month extension)

HUNTINGDON RESEARCH CENTER (PH43-68-1516)

Title: To Prepare and Test Reference Reagents to Recognized Human and Animal Mycoplasma Types

Project Director: Dr. Theodore R. Carski

Project Officers: Dr. Joseph Tully and Mrs. Sylvia Cunningham

Objectives: To produce and test mycoplasma reference reagents to serve as reference standards for research on mycoplasmas.

Major Findings: Production of 28 mycoplasma seed reagents and corresponding antiserum of both human and non-human origin has been completed. Nine human mycoplasma antisera are finished (NOTE: the production of antisera of non-human origin was funded by the Division of Biologics Standards. The production of all mycoplasma reagents is complete)

Significance to NIAID Program and Bio-Medical Research: Mycoplasma reagents are essential in bio-medical research due to their significant role in diseases of man and animals and because of the frequency with which they occur as contaminants in tissue culture lines, media and biological products.

Proposed Course of Project: Terminated November 1970

Date Contract Initiated: June 26, 1968

Current Annual Level: No FY 71 funds

ABBOTT LABORATORIES (PH43-67-1355)

Title: Production of Reference Reagents to 56 Prototype Rhinoviruses

Project Director: Mr. George S. Marquis

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to 56 prototype rhinoviruses.

Major Findings: The contractor has completed the preparation of master seed to 56 prototypes. Forty five final vial seed virus reagents have been approved for distribution. Five have been rejected and six are currently under test. Fifty six bulk antisera have been produced with two being rejected and two others currently under test. Twenty two sera have been processed and are currently being tested by the certification laboratory.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated June 1971

Date Contract Initiated: June 26, 1967

Current Annual Level: $6,979 (two month extension)

UNIVERSITY OF VERMONT (PH43-67-1188)

Title: To Test, Identify and Certify Rhinovirus Reference Reagents

Project Director: Dr. Charles A. Phillips

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To authenticate fifty-six Phase I rhinovirus reagents produced by Abbott Laboratories for identity, potency and purity. They will also authenticate Phase II rhinovirus reagents and any Phase I remakes to be produced by five production contractors.

Major Findings: To date forty-nine final vial antigens have been tested and approved for distribution while forty-two bulk antisera have been approved for packaging. Five seed viruses and two bulk antisera have been rejected because of low titer or contamination.

During the past year twenty-nine bulk antisera were tested and approved for packaging while seven final vial seed viruses, twenty-six final vial antisera and two bulk antisera are on test.

Significance to NIAID Program and Bio-Medical Research: The contract furnishes authentication and purity confirmation on rhinovirus reagents being or to be produced by other independent contractors. The comprehensive certification testing will enhance the value of the rhinovirus reagents as reference standards.

Proposed Course of Project: The five contracts for the production of thirty-four Phase II rhinoviruses and any Phase I remakes are currently under negotiation and as long as a contract is in effect for the production of rhinovirus reference reagents, the service of this contractor will be needed.

Date Contract Initiated: June 1967

Current Annual Level: No FY 71 funds

UNIVERSITY OF WISCONSIN (NIH-71-2111)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Elliot Dick

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to 10 prototype rhinoviruses.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated April 1972

Date Contract Initiated: April 1971

Current Annual Level: $49,800

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (NIH-71-2112)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Jack Schieble

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to five prototype rhinoviruses.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated March 1972

Date Contract Initiated: March 1971

Current Annual Level: $38,170

ABBOTT LABORATORIES (NIH-71-2114)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Mr. George S. Marquis

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to 20 prototype rhinoviruses.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated in April 1972

Date Contract Initiated: April 1971

Current Annual Level: $108,780

UNIVERSITY OF VIRGINIA (NIH-71-2113)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Jack Gwaltney

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to five prototype rhinoviruses.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated April 1972

Date Contract Initiated: April 1971

Current Annual Level: $47,212

SOUTHWEST FOUNDATION FOR RESEARCH AND EDUCATION (NIH-71-2110)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. S. S. Kalter

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to six prototype rhinoviruses.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated April 1972

Date Contract Initiated: April 1971

Current Annual Level: $38,745

LEDERLE LABORATORIES (NIH-69-2043)

Title: Production and Testing of Myxovirus Reference Reagents

Project Director: Dr. Paul J. Vasington

Project Officers: Dr. Robert M. Chanock and Mr. Robert M. Pennington

Objectives: To produce, test and package seed virus reagent to four myxovirus types and antisera to five myxovirus types.

Major Findings: The Contractor has produced and tested the seed and antisera for all assigned myxoviruses. Six of the nine reagents have been approved for distribution and the remaining three are currently being tested.

Significance to NIAID Program and Bio-Medical Research: This contract will furnish nine reference reagents to help fill the need of NIH grantees, intramural scientists, contractors and other eligible recipients of reference reagents.

Proposed Course of Project: Terminated February 1971

Date Contract Initiated: June 1969

Current Annual Level: No FY 71 money

CENTER LABORATORIES (NIH-70-2226)

Title: Extraction of Antigen E from Dwarf Ragweed Ambrosia Elatior

Project Director: Mr. J. George Center

Project Officers: Dr. Charles Kirkpatrick and Mr. Robert M. Pennington

Objectives: To extract, process and package purified antigen E suitable for in vivo and in vitro immunological studies.

Major Findings: The bulk antigen E reagent had to be reprocessed after the initial purification. The purity was considerably below that stipulated in the specifications. A second purification was recently completed and shown to meet the criteria for potency and specificity. The purified antigen E is currently being packaged for distribution. It is anticipated that this product will be distributed under an NIAID sponsored IND.

Significance to NIAID Program and Bio-Medical Research: The Contractor will make available a highly purified and characterized homogeneous allergen which may be used as a standard preparation for ragweed. Antigen E is chemically defined so that one could modify it and retest its antigenicity. There are at least thirteen ragweed antigens and only three have been characterized; antigen E, antigen K and antigen Ra 3.

Proposed Course of Project: To be terminated May 1971

Date Contract Initiated: June 1970

Current Annual Level: $25,000-$35,000 (dependent on number of ampoules filled)

NORTH AMERICAN BIOLOGICALS, INC. (NIH-71-2100)

Title: Production of AAV Reagents

Project Director: Dr. Robert R. Rafajko

Project Officers: Dr. James Rose and Mr. Robert M. Pennington

Objectives: To produce, test and package seed virus reagent and antisera to 4 adeno-associated virus reagents.

Major Findings: None. Project recently initiated.

Significance to NIAID Program and Bio-Medical Research: This contract will furnish four adeno-associated virus reagents which are an essential part of the total adenovirus reagent program due to their adventitious nature in adenovirus seed.

Proposed Course of Project: To be terminated April 1972

Date Contract Initiated: April 6, 1971

Current Annual Level: $18,700

Zonal Centrifuge Program

AEC-NIAID

Title: Molecular Anatomy (MAN) Program (Rockville and ORNL)

Project Director: Dr. Norman Anderson (Director, ORNL)

Project Investigator: Dr. John Gerin (Resident Director, Rockville)

Project Officers: Dr. Robert J. Byrne, Mr. Robert M. Pennington and
Mr. Merle Callahan

Objectives: MAN (ORNL) - To develop biophysical and engineering methodology applicable to NIAID Programs. MAN (Rockville) - 1) To conduct collaborative research with NIAID Intramural scientists and collaborative research contractors; 2) To apply separation systems developed through MAN programs at Oak Ridge; 3) To train NIAID intramural scientists and collaborative research contractors in separation techniques.

Methods Employed: The MAN activity (ORNL) is engaged in the development of rotor systems and other biophysical instrumentation applicable to the NIAID programs. The Rockville facility evaluates centrifuge and biophysical instrumentation systems developed at ORNL and Rockville and applies them to specific projects of interest in the extramural and/or intramural programs of NIAID.

Major Findings: Studies of Australia Antigen: 1) Acrylamide gel electrophoresis studies indicate that Au consists of at least two proteins one of which seems to be a common or "group-specific" antigen; 2) Isoelectrofocusing of purified Au; Purified Au yeilds two major and one minor component by both molecular weight and isoelectric analysis; 3) "Fingerprint" analyses have shown that the different proteins of Au have similar amino acid sequences, they are different in lysine and arginine residues or that the tryptic digest was incomplete. Amino acid analysis is presently being determined for the characterization of the various subtype specificities of Au; 4) Sedimentation coefficients of Au give a S°_{20}, W=81.1 which is a reliable value for the 22mm spherical form of the Au particle; 5) Studies to detect RNA dependent DNA polymerase activity in Au antigen purified by isopycnic banding and rate zonal procedures have been negative; 6) Structural studies of Au antigen suggest that Au possess hemagglutinin activity which results from the exposure of an antigen, possibly an internal antigen, during the dissociation of the spherical particle. Studies are now in progress to dissociate the Au particle to the level of protein subunits; 7) It would appear that there are four antigenic specificities related to Au; all Au specimens possess a common or "group specific" "a" antigen. The Au described in the above studies is an Au-a-d subtype and antisera prepared against the antigen possess two specificities, anti-a and anti-d. Reagents for other specificities are being prepared or will be prepared in the near future; 8) Collaborative work is being pursued with Dr. Saul Krugman by purifying several units of MS-2 serum for use in indirect hemagglutinin assay in support of his present studies at Willowbrook School.

Other Studies Currently in Progress: 1) Neuraminadase assay - a continuous spectrophotometric method for neuraminadase activity is being developed. It is hoped that the method will be a simple assay for the presence of neuraminadase and, by inhibition, for specific antibodies against neuraminadase; 2) X-32 Influenza Vaccine - a bulk recombinant vaccine has been prepared. The X-32 recombinant contains an equine hemagglutinin and an A2/Hong Kong neuraminadase; 3) Progressive Multifocal Leucoencephalopathy (PML) is currently being studied in the hopes of obtaining enough purified material to produce hyperimmune serum in guinea pigs for the identification of this human pathogen; 4) The growth of Respiratory Syncytial Virus in spinner cultures is currently being evaluated. The use of spinner cultures should result in the production of rather large quantities of RS virus allowing for more suitable planned biochemical studies of this virus. Biophysical studies of RS virus, the wild type and ts-2 mutant are currently in progress. Labeled and released ts-2 virus is undergoing purification for possible use in a radio-immunoassay and for biochemical studies of its protein.

Significance to NIAID Program and Bio-Medical Research: The development and utilization of these separation systems provides a unique opportunity for the purification of viruses and virus subunits which will have application in the development of more effective vaccines. The program also affords the opportunity for training of NIAID and Collaborative contract scientists.

Proposed Course of Project: Due to the nature of the project and the rental agreement of the satellite facility it is estimated that the project will continue for at least five years at $250,000/annum based on the availability of funds.

Date Contract Initiated: April 24, 1964

Current Annual Level: $250,000

Publications

1. Gerin, J. L., Holland, P. V., Purcell, R. H.: Australia Antigen: Large-Scale Purification from Human Serum and Biochemical Studies of its Proteins. J. of Virology (In Press)

2. Schmidt, N. J., Melnick, J. L., Wenner, H. A., Ho, H. H., and Burkhardt, M. A.: Evaluation of Enterovirus Immune Horse Serum Pools for Identification of Virus Field Strains. Bull. Wld. Hlth. Org. (In Press)

3. Gerwin, B. I., Todaro, G. J., Zeve, V., Scolnick, E. M., and Aaronson, S. A.: Separation of RNA-Dependent DNA Polymerase Activity from the Murine Leukaemia Virion. Nature. 228: 435-438, 1970

4. Melnick, J. L., and Hampil, B.: WHO Collaborative Studies on Enterovirus Reference Antisera. Third Report. Bull. Wld. Hlth. Org. 42: 847-863, 1970

5. Western Hemisphere Committee on Animal Virus Characterization: Animal Reference Virus Recommendations. Am. J. Vet. Res., 31: 1915-1925, 1970.

TRANSPLANTATION AND IMMUNOLOGY PROGRAM

1. Summary

Results of the evaluation of histocompatibility typing have demonstrated the need for multiple serologic tests to identify the antigens involved in the rejection of transplanted organs. Program contractors have recently shown platelet complement fixation and the capillary migration tests to be of importance in the identification of histocompatibility antigens. The results of typing may, on occasions, be improved by the utilization of donor lymph node or kidney cells. The quality and value of reagents being distributed is insured by coordinated testing by contract quality control laboratories and regional typing evaluation facilities. Considerable program effort was devoted to improving techniques for freeze drying of reagents. Further refinements have been made in the procedures for storing, distributing and utilizing scarce sera.

The Transplantation and Immunology Branch provides contracts to evaluate drugs and antilymphocyte serum in immunosuppression. With promising results obtained in subhuman primate skin graft studies, a program has been undertaken to produce large quantities of rabbit antihuman thymocyte serum which will be used in a clinical evaluation of immunosuppression.

Another essential part of the Transplantation Program is the preservation of cells and organs. The Program supported studies in pulsatile perfusion of kidneys and long-term hyperbaric cryopreservation. In addition, a method has been developed for the frozen preservation of lymphoid cells.

The Program is also seeking to improve means of obtaining purified HL-A antigens which might be used as a means to induce tolerance in potential recipients of organ transplants and to immunize laboratory animals to produce high titer monospecific antisera needed for histocompatibility typing.

Several contracts study the immunologic phenomena associated with organ rejection in the patient. These indicate there is an increase in rheumatoid-like factor in transplant recipients and that antigen antibody complexes may be deposited in the kidneys and other organs of graft recipients.

One of the most important functions of the Transplantation and Immunology Program is to collect and disseminate information on organ transplants and the efficacy of tissue matching. The Program supports registries, sponsors workshops and scientific meetings and, through the publication of contractor's reports and the issuance of catalogs, typing manuals and periodic statistical reports, provides a valuable resource of scientific and technical information.

2. Program Status Report

During the period July 1, 1970 to June 30, 1971, the Branch provided approximately $1.8 million in contract funds for the support of transplantation and immunology programs. The research and development efforts of the Branch can be considered in six general categories.

I. Histocompatibility Typing Facilities and Immunological Reagents

Since the collaborative transplantation immunology program was established in 1965, much progress has been made in developing the methodology of histocompatibility typing and the reagents necessary for this work. The current emphases of the fifteen contracts in this field are to evaluate the efficacy of typing in selecting unrelated donors and recipients for organ transplantation; to provide a continuing supply of high-quality typing sera both in bulk and in micro-quantities on a standardized tray; to explore new typing techniques that are potentially simpler or more accurate than the most widely used method; to conduct research directed at discovering new specificities; and to provide other reagents used in histocompatibility research. Fifteen contracts have been supported to work in one or more of the above areas, with total funding of $673,371.

The evaluation of the efficacy of typing in selecting unrelated organ donors and recipients (carried out in eight laboratories) has proved to be a complex problem. Analyses of the data based on a simple count of the mismatched antigens have yielded conflicting results due, in part, to the difficulties involved in collecting accurate clinical data. The consensus to date is that there exists a correlation between closeness of matching and renal graft function. Early results from a more sophisticated analysis, based on the number of shared haplotypes between donors and recipients, have been very encouraging. One contract is analyzing the usefulness of typing in bone marrow transplantation.

The NIAID typing serum bank currently contains over 900 sera with a total volume of 166 liters. Additions to the bank in FY 71 included 141 (25,156 ml.) sera obtained by contract from nine laboratories, 29 (5,900 ml.) donated by users of the bank and 28 (3,224 ml.) purchased outright by the Branch. The additions include 33 sera judged by WHO investigators as among the best in the world. Quality control analyses of the sera prior to their procurement were performed by two typing contractors; this system has increased the overall quality of the additions to the bank. Usage of the bank remained heavy, with 492 shipments (20,000 ml.) dispensed to 200 investigators. In addition, significant improvements in the clinical typing tray program were made. Forty-one sera were carefully selected for use on a tray to be kept constant over a two-year period; typing results are collected from each tray and will be used in an expansion of the Branch's effort to evaluate the efficacy of typing. Approximately 1,000 trays per month are made on one contract and are being used by U.S. clinical typing programs.

New techniques of typing were studied on seven contracts. The use of kidney cells has been found to provide a valuable additional histocompatibility cross-match test and they may help detect additional antigens in clinical typing. The platelet complement fixation test continued to gain stature as a simple, easily readable typing technique, and the capillary migration technique has been established as a very sensitive technique. Automation of the cytotoxicity test has been accomplished and is on the verge of being used routinely in one laboratory. Neutrophil-specific antigens have been discovered and are being studied in bone marrow transplantation donor-recipient pairs. Better methodology and reagents have resulted in the discovery of new antigens; several of the typing contracts have made contributions in this area.

Two contracts have produced reagents other than typing sera used in histocompatibility research. Human complement components and inhibitors are distributed to qualified investigators, and mouse spleens have been provided in large quantities as starting material for the production of soluble mouse histocompatibility antigen (described in Section IV).

II. Studies of Immunosuppression

Five contracts totaling $476,991 provide funds to evaluate potentially active immunosuppressive drugs and sera in animals and patients. These help to develop techniques for the prevention or the abrogation of graft rejection. Cyclophosphamide and a variety of new antineoplastic drugs are currently being investigated. Studies to date in animals and patients indicate that cyclophosphamide is an effective agent for the prevention of graft rejection in bone marrow transplantation. Other antitumor agents (methotrexate, cytosine arabinoside and L-asparaginase) were used individually and with antilymphocyte serum and have been shown to be effective in preventing first and second set graft rejection. Important methodology has been developed to evaluate immunosuppressives.

Antilymphocyte serum (ALS) and purified antilymphocyte globulin (ALG) are of current interest as means of preventing and reversing rejection. Over 35 percent of the U.S. transplantation programs utilize these preparations. Unfortunately, few controlled studies have been conducted to evaluate the efficacy of these products. The Transplantation and Immunology Branch has established the only Western Hemisphere facility for the mass in vivo testing of ALS and ALG. This contract has screened more than 120 preparations, many of which are currently being used in the clinic. The two most effective antigens for the production of ALS are thoracic duct and thymus cells; ALS raised in rabbits is superior to that produced in horses in the prolongation of Rhesus and chimpanzee skin grafts. A program is now underway to produce sufficient quantities of this material to support a 50-patient clinical trial. The high cost of subhuman primate testing has prompted the search for effective in vivo techniques of assaying ALS and ALG.

Preliminary studies indicate that rabbit antihuman thymocyte serum is effective only if it exhibits a high in-vitro lymphocytotoxic titer. This technique is being utilized to exclude from subhuman primate testing non-effective rabbit antisera.

III. Organ Preservation Studies

A transplantation program is heavily dependent upon the availability of viable organs. Because of the many difficulties involved in the long-term preservation of organs, current kidney-sharing programs maintain a pool of potential recipients from which one is selected (via histocompatibility matching) to receive a specific kidney immediately after the organ becomes available. An NIAID contract (no FY 1971 funds involved) completed its study of the pulsatile perfusion of synthetic solutions through kidneys, a system designed to provide short-term preservation for the hours needed to complete the histocompatibility matching.

The chief disadvantage of the above system is that it keeps a large number of patients waiting to receive a transplant; if long-term kidney preservation were possible, a pool of organs could be maintained from which one could be selected for a specific patient as soon as that patient needed such a transplant. An NIAID contract, ($161,000) has been responsible for the development of a multifaceted attack on the problems of long-term organ preservation. Accomplishments thus far include the development of cryoprotective medium more effective than those currently used; the application of methods of treatment used against frostbite to the resuscitation of frozen organs; the development of an enzyme assay for organ damage incurred during freezing and thawing and of a microwave thawing device which rapidly and evenly thaws a kidney; and the use of hyperbaria to decrease the formation of extracellular ice during the freezing process.

IV. Antigen Studies

The Institute provides $182,363 for the support of two contracts for the study of soluble murine and human histocompatibility antigens. Several methods of antigen solubilization and fractionation are being employed to produce active stable fractions which are then assayed by a variety of in vitro and in vivo techniques. These antigen fractions are also provided to NIH intramural, contract and grantee investigators who aid in the investigation of these products for their immunogenic and tolerogenic properties. The ultimate goal of this program is to determine the feasibility of using soluble histocompatibility antigens to induce tolerance in humans, so that histoincompatible tissue may be successfully utilized, thereby circumventing the problems of graft rejection. These soluble antigens can be employed to produce monospecific histocompatibility typing serum for the NIH serum bank.

V. Immunological Phenomena

Two contracts totaling $163,398 evaluate the immunological changes in the tissues and serum of patients and animals who have received organ transplants and/or immunosuppressive treatment. There is an increase in rheumatoid-like factor in the serum of transplant recipients and titers usually are higher in patients who have incompatible grafts. The mechanism of hyperacute graft rejection and its immunological process is under investigation. Immunohistological changes in transplanted and rejected renal grafts are being investigated to assess whether graft rejection is due to recurrent nephritis, to the deposition of circulating immune complexes in the renal tubules or glomeruli, or to an immunologic histoincompatibility reaction. Studies involving antilymphocyte globulin (ALG) preparations used in immunosuppressive therapy have revealed that 70 percent of those preparations derived from solid lymphoid organ lymphocytes contained anti-glomerular basement membrane (GBM) antibodies. Two human liver graft recipients who were treated with equine ALG that contained anti-GBM had equine immunoglobulin bound to their glomerular basement membranes.

VI. Statistical Studies and the Dissemination of Scientific Information

The Branch supports part of the American College of Surgeons/National Institutes of Health contract for the collection of transplantation information. The Branch Chief is the Project Officer for the ACS/NIH Kidney Registry and Head of the NIH Transplantation Committee which provides over-all supervision for this contract.

The Transplantation and Immunology Branch conducts its own program for the collection of fundamental information on transplants. On an annual basis, serum users are asked to provide data indicating the numbers of transplants of different organs conducted by the various hospitals for whom they provide typing services. The data is collated and used by the Branch in assessing priorities for the distribution of serum, and is distributed to other serum users and sent to the ACS/NIH Registry. Experience in the past has shown that this mechanism has been far more successful in obtaining transplantation information than other registries. For example, during 1970, serum users indicated that 2,920 kidney transplants had been performed in 301 hospitals around the world; during this same period the ACS/NIH Registry collected data on approximately 2,000 renal transplants in 175 institutions. As part of its information program, the Branch prepares an annual updating of the Tissue Typing Catalog. The last issue of this catalog was distributed in November 1970 to approximately 800 users. This catalog, which is over 600 pages in length, contains information on 900 sera which are available from the Institute's bank. It lists the availability of serum and a great deal of information concerning the specificity, utility, titers and the frequency of reaction with the various racial groups. It is the only such document available for use by transplantation immunologists.

The Manual of Tissue Typing Techniques was revised during the last fiscal year. This contains up to the minute information on the various tests which may be used for histocompatibility matching, and is distributed to investigators and used in the tissue typing courses. The Branch also provides catalogs of other reagents, including H-2 (mouse) antisera, complement components, and soluble antigen fractions.

Contract funds provided partial support for the Third International Congress of the Transplantation Society held at The Hague, The Netherlands, in September 1970. This support aided in the organization of the conferences and provided travel funds for 40 NIAID grantees and contractors who presented original papers at the meeting.

The Branch also distributes information to scientists by means of periodic bulletins, and aids in the organization and operation of histocompatibility workshops.

3. CONTRACT NARRATIVES

ALBERT EINSTEIN COLLEGE OF MEDICINE, YESHIVA UNIVERSITY (PH43-68-1295)

Title: Preparation of soluble purified H-2 isoantigens

Contractor's Project Director: Dr. Stanley G. Nathenson

Project Officers: Dr. John G. Ray, Jr and Mr. C. Eugene Harris

Objectives: This contract provides soluble mouse H-2 antigens to selected qualified investigators who are interested in applying these antigens in studies of immunogenicity and immunological tolerance. This contract provides the contractor with funds for storage and distribution of these antigens and to improve the efficiency of the solubilization and purification processes.

Major Findings: This investigator has processed approximately 160,000 mouse spleens from six different strains of mice to acquire soluble H-2 histocompatibility antigens in various stages of purity. Thus far, twenty investigators have utilized approximately 1,200 milligrams of these soluble antigenic fractions in support of their research activities. These soluble H-2 alloantigens have been shown to be immunogenic as well as modify graft-versus-host reactions.

Significance to NIAID Program and Bio-Medical Research: Basic immunogenetic knowledge concerning the immunogenicity and tolerogenicity of mouse H-2 soluble histocompatibility antigens and their relationship to accelerating and inhibiting graft rejection will be enhanced by this contract. These studies are in concert with similar studies of human HLA soluble antigen production and eventual immunogenicity and tolerogenicity studies in experimental animals and humans. The ultimate goal is to develop methods to abrogate the graft rejection process and permit permanent graft survival.

Proposed Course of Project: Continuation through June 23, 1972

Date Contract Initiated: June 24, 1968

Current Annual Level: $89,370

ALBERT EINSTEIN COLLEGE OF MEDICINE (NIH69-2071)

Title: Quality control and reference laboratory for leukocyte antisera

Contractor's Project Director: Dr. Parviz Lalezari

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is screening potential donors for antisera useful in neutrophil typing, and is studying the reactions of these sera with neutrophils and lymphocytes. Neutrophil typing will be done in conjunction with bone marrow transplants.

Major Findings: Emphasis has been placed on the study of neutrophil-specific antigens, with the antibodies involved in neonatal neutropenia serving as the starting point of investigation. Four different neutrophil specificities have been found, using an agglutination technique in the presence of EDTA, and antisera to three of these specificities have been contributed to the Serum Bank. Two of the antigens appear to be alleles. Leukocyte agglutinins have been found in 26 patients post renal transplantation; these agglutinins are being analyzed for the presence of neutrophil-specific antibodies.

Significance to NIAID Program and Bio-Medical Research: The major contribution of this contract has been in the inception of the study of neutrophil-specific antigens. Such antigens are apparently involved in neonatal neutropenia, and may well be of great importance in determining the success or failure of bone marrow transplants, which are now being performed to correct severe neutropenia of various etiologies. Thus, the careful definition of the genetics of neutrophil antigens and the further determination of their pathological significance are questions of pressing clinical importance. The activity of antibodies made to neutrophil antigens against renal or other solid organ transplants is a question of great clinical importance that is also under study.

Proposed Course of Project: Continuation through December 26, 1971

Date Contract Initiated: June 27, 1969

Current Annual Level: **Prior Year Support**

UNIVERSITY OF CALIFORNIA, LOS ANGELES (PH43-65-994)

Title: Evaluation of the role of leukocyte typing in kidney transplantation

Contractor's Project Director: Dr. Paul I. Terasaki

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is evaluating the efficacy of leukocyte typing in clinical transplantation by typing for transplants in the Los Angeles area, using the lymphocytotoxicity, the platelet complement fixation and the mixed lymphocyte culture techniques. He evaluates samples of typing antisera from the Serum Bank and prepares the NIH typing trays, containing sera in lambda quantities covering the full range of specificities, for distribution by the Branch to clinical leukocyte typing laboratories. He is conducting a screening program for valuable antisera.

Major Findings: In recent years, this contractor has typed for transplant programs throughout the country, using lymphocytes mailed to his laboratory. He also has collected clinical data, using the questionnaire method. In September 1970, he presented data on typing-graft survival correlations obtained from 1200 unrelated renal transplants. The differences in the graft survival of matched and unmatched transplants were not significant. Evidence to the contrary has been presented by other contractors and foreign laboratories, and the question remains unresolved; however, the Branch has decided that the mass collection of data by a single laboratory is not the method that will obtain the highest quality clinical and typing data. Thus, this project is now acting as a Los Angeles regional typing facility. The NIH typing trays, produced by this contract, have become the major source of quality typing sera for clinical typing laboratories in the U.S., and, with the new standardized tray, this program will serve as a source of highly comparable typing data over a two-year period. Quality control work on Serum Bank sera done by this contractor has aided substantially in improving the Bank's stock of reagents. The collection of platelet complement fixations and mixed lymphocyte culture typing data in conjunction with the standard lymphocytotoxicity results is underway and will aid greatly in assessing the value of the two newer techniques. The serum screening program has yielded some valuable sera and has been instrumental in the definition of new specificities.

Significance to NIAID Program and Bio-Medical Research: The support of this laboratory as a regional typing laboratory is part of the Branch's plan to evaluate the efficacy of histocompatibility matching by collecting typing and clinical data from the several large U.S. transplant programs. The provision of the typing trays is the major means of rendering the typing data from different centers comparable. The study of new typing techniques is part of an effort to make typing more accurate and rapid; these goals are particularly important in clinical typing.

Proposed Course of Project: Continuation through September 1, 1971

Date Contract Initiated: June 22, 1965

Current Annual Level: $153,000

CLEVELAND CLINIC FOUNDATION (PH43-68-1298)

Title: Histocompatibility testing of donors and recipients of organ grafts

Contractor's Project Director: Dr. William Braun

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is evaluating the efficacy of tissue-typing in matching donors and recipients of renal grafts in the Cleveland area, is providing histocompatibility antisera for the TIB Serum Bank, and serves as a quality-control laboratory for testing typing reagents.

Major Findings: This contractor has developed an excellent facility for the evaluation of the efficacy of tissue-typing in a short period of time. He has developed a high-quality panel of sera for typing purposes, which he uses in conjunction with the NIH tray, and in 1970, typed for more kidney transplants (82) than any other U.S. laboratory. Data accumulated thus far from related donors and recipients support that of other laboratories showing better graft survival with matched as opposed to mismatched renal transplants. Early results of an analysis of unrelated transplants (based on the number of shared haplotypes) provide support for the efficacy of matching. The serum procurement program has produced valuable antisera for the TIB Serum Bank. He has analyzed over 300 sera for the Serum Bank in the past nine months.

Significance to NIAID Program and Bio-Medical Research: This contract is a valuable part of the Branch's effort to assess the efficacy of tissue-typing in selecting organ donors and recipients, and has been productive in supplying new sera, essential for the operation of the Serum Bank. The quality control work is an essential part of the Branch's efforts to constantly improve the quality of the Serum Bank's typing reagents.

Proposed Course of Project: Continuation through December 27, 1971

Date Contract Initiated: June 28, 1968

Current Annual Level: $70,097 (18 month period) - $17,149 FY 1971 funds.

COMMUNITY BLOOD COUNCIL OF GREATER NEW YORK (PH43-67-1334)

Title: Histocompatibility testing of donors and recipients of organ grafts

Contractor's Project Director: Dr. Fred H. Allen, Jr.

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is evaluating tissue-typing as a means of selecting donors and recipients of organ grafts in the New York City area.

Major Findings: Approximately 900 leukocyte typings have been done in 1970, using primarily a microlymphocytotoxicity technique. Preliminary data on the limited number of transplants performed suggest a longer survival of well-matched grafts, if the data is analyzed on the basis of the number of haplotypes shared by the donors and recipients. Data collected on the red cell groups and serum proteins of the individuals typed have ruled out close linkage of most of the common RBC and histocompatibility antigens.

Significance to NIAID Program and Bio-Medical Research: This contract provides a unique opportunity to correlate red cell and serum protein groups with leukocyte-typing in the selection of donors and recipients of organ grafts. The study of the efficacy of typing in selecting unrelated graft donors and recipients is yielding encouraging results with the new haplotype analysis; statistical significance of the differences is anticipated during the current year as more transplants are performed.

Proposed Course of Project: Continuation through June 28, 1972

Date Contract Initiated: June 29, 1967

Current Annual Level: $63,208

HARVARD UNIVERSITY (NIH-69-90)

Title: Extraction, characterization, and testing of HL-A antigens and immunogenetic studies of the HL-A system.

Contractor's Project Director: Dr. Paul S. Russell

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor is to produce water soluble HL-A antigens from human tissue, and tissue type these extracted antigens to characterize their HL-A specificity. After producing these HL-A antigens, he is to produce specific antisera against these antigens.

Major Findings: Three HL-A alloantigenic partially purified preparations have been produced by exposure of tissue cells to a brief, controlled burst of low intensity sound. These antigens show specific cytotoxic inhibition of sera from the NIAID Serum Bank when typed against lymphocytes denoting the antigenic determinants HL-A 1, 2, 3, 7, and 8. It has been possible to assay the strength of various HL-A antisera by using these antigenic preparations, but unfortunately these antigens are relatively unstable and rapidly deteriorate when stored at -70° and -60°C. The half-life of activity of these antigens is approximately six weeks under storage conditions and one day at room temperature.

Significance to NIAID Program and Bio-Medical Research: Human histocompatibility locus antigens (HL-A) are needed to provide qualified investigators with relatively pure antigenic material for immunogenetic studies which will relate the relative importance of these antigens in the rejection process. They can be employed to initiate feasibility studies of inducing tolerance in a graft recipient so that he will not reject his graft because of histoincompatibility to one or more HL-A antigens. These soluble antigens may be used to produce much needed monospecific HL-A antisera for the NIAID Serum Bank. They may also be used as antigenic typing reagents for *in vitro* testing of HL-A identity in serum and in tissue. The problem of stability must however be resolved before fruitful information can be obtained.

Proposed Course of Project: Continuation through May 8, 1972.

Date Contract Initiated: April 9, 1969

Current Annual Level: $92,993

UNIVERSITY OF CHICAGO (PH43-69-27)
UNIVERSITY OF IOWA (NIH-70-2011)

Title: Study of the capillary migration assay

Contractor's Project Director: Dr. John S. Thompson

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is further developing the capillary migration method of leukocyte typing, and is correlating its results with the cytotoxicity and one-way mixed lymphocyte culture techniques. He also is screening for sera particularly useful in the capillary migration technique.

Major Findings: The contractor has refined this technique so that only lambda quantities of sera are required, and leukocytes stored for up to two weeks in formalin can be used. Mixed leukocytes or pure lymphocyte preparations can be used interchangeably. With available typing sera, the technique is too sensitive for clinical leukocyte typing, but is considered excellent for cross-matching and for leukocyte genetics research; antigens are detected by capillary migration in cells that are non-stimulating by mixed lymphocyte culture and negative by cytotoxicity. The technique is functional with the one anti-neutrophil sera tested and also with two non-cytotoxic sera collected from patients following severe transfusion reactions; it is considered a valuable tool in detecting non-HL-A leukocyte specificities that may have considerable relevance to transplantation.

Significance to NIAID Program and Bio-Medical Research: This technique has its major usefulness in histocompatibility cross-matching and in research on the genetics of leukocyte antigens. It has great potential as an aid in defining new leukocyte antigens and in separating out those important in transplantation, and appears to be applicable to both lymphocyte and neutrophil antigens.

Proposed Course of Project: Continuation through June 30, 1971.

Date Contract Initiated: Univ. of Chicago - August 26, 1968 - terminated August 25, 1969. Continued at University of Iowa, beginning November 28, 1970

Current Annual Level: **Prior Year Support**

IRWIN MEMORIAL BLOOD BANK OF THE SAN FRANCISCO MEDICAL SOCIETY (PH43-68-945)

Title: A regional leukocyte phenotyping center in a community blood bank to evaluate histocompatibility typing

Contractor's Project Director: Dr. Herbert A. Perkins

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is conducting histocompatibility typing of leukocytes and kidney cells of prospective donors and recipients in the San Francisco area, and is conducting a serum screening program to provide histocompatibility typing antisera for the Serum Bank.

Major Findings: Using the fluorochromasia technique of Bodmer, approximately 710 leukocyte typings were performed in 1970 in connection with the largest single U.S. kidney transplant program. The data underscore the usefulness of typing in related donors and recipients; with cadaver donor transplants, a recent retyping of lymphocytes (preserved by freezing) from transplant donors and recipients (matched in the past by cruder methods of typing) has yielded support for the efficacy of histocompatibility matching. Preliminary results indicate that the typing of both kidney cells and lymphocytes results in the more frequent detection of HL-A4, 7, and 8 than would the typing of only one cell type. Valuable typing sera have been discovered through the serum screening program.

Significance to NIAID Program and Bio-Medical Research: This contractor's affiliation with a very active transplant program facilitates the comparison of the typing of leukocyte and kidney cells in terms of the clinical outcome of renal grafts, a question of great importance that currently is unanswered. The procurement of typing serum is a task essential to the continuated operation of the Serum Bank.

Proposed Course of Project: Continuation through October 26, 1971

Date Contract Initiated: March 27, 1968

Current Annual Level: Prior Year Support

THE JACKSON LABORATORY (PH43-66-483)

Title: The production of monospecific H-2 antisera and specific mouse spleens.

Contractor's Project Director: Dr. George Snell and Dr. Marianna Cherry

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor is to maintain a mouse H-2 alloantiserum bank which stores and distributes these antisera to NIAID-authorized requesters. He is to periodically check these sera for potency of reactivity and specificity recharacterization. In addition, this contractor will prepare, store and distribute approximately 60,000 mouse spleens to another contractor, Dr. Stanley Nathenson, for extraction of soluble mouse H-2 antigens.

Major Findings: This contract has supplied 72 different scientific investigators with approximately 4,700 ml of mouse H-2 alloantisera for use in studying the immunogenetics of graft rejection and the histocompatibility loci. Similarly, approximately 160,000 mouse spleens have been shipped to Dr. Stanley Nathenson. There seems to be good retention of antibody activity in the banked antisera since no notable differences have occurred after six months evaluation among antisera stored lyophilized, at -20°C, or at -60°C. Upon rechecking the specificity of the antisera using a different technique, a number of unpredicted antibodies have now been detected which will modify and improve the original "H-2 Chart" mouse strain combinations needed for making H-2 antisera.

Significance to NIAID Program and Bio-Medical Research: These reagents are the basic immunogenetic ingredients from which knowledge will be enhanced concerning the immunogenicity and tolerogenicity of the mouse H-2 histocompatibility antigens. This has a direct application to the elucidation of the HLA antigenic system in human transplantation and its role in the rejection phenomenon.

Proposed Course of Project: Continuation through April 30, 1974

Date Contract Initiated: June 1, 1966

Current Annual Level: $235,000 (three years)

KUAKINI MEDICAL RESEARCH INSTITUTE (PH43-66-923)

Title: Anti-leukocyte serum procurement and population studies for lymphocyte typing in Hawaii

Contractor's Project Director: Dr. Mitsuo Yokoyama

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contractor provided unique human leukocyte antisera to the NIAID Serum Bank by conducting immunogenetic studies of the Hawaiian multiracial population and by obtaining quantities of quality antisera from multiparous women.

Major Findings: This contract was phased out during 1970; during the terminal year, the contractor continued to analyze data on serum obtained and to study the mixed Hawaiian population. Sera produced under this contract have proved to be useful reagents for clinical typing. Several of these are currently used on the standard NIH histocompatibility tray.

Significance to NIAID Program and Bio-Medical Research: This Hawaiian population was a unique source for several good sera which are rarely found in the mainland Caucasian population. With the development of more widespread laboratory facilities in many countries, sera for these specificities are becoming more plentiful and are currently being acquired by the bank by donation or direct purchase.

Proposed Course of Project: Terminated December 29, 1970

Date Contract Initiated: June 30, 1966

Current Annual Level: Prior Year Support

UNIVERSITY OF LEIDEN (PH43-65-992)

Title: Search for specific HL-A sera and improvement of histocompatibility techniques

Contractor's Project Director: Dr. Jon J. van Rood

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract seeks to further evaluate the efficacy of platelet complement fixation and agglutination techniques as a means of selecting donors and recipients for organ transplants and to evaluate the efficacy of histocompatibility matching as a means of preventing graft rejection. Further, the contract provides funds to search for and collect quantities of antisera containing complement fixing antibodies against specificities.

Major Findings: The contractor has developed a panel of cell donors which he uses in a program to find monospecific serum and to discover new specificities for the platelet complement fixation test. He has deposited 9,875 cc of unique histocompatibility typing serum in the bank during the past year. Comparisons are being done in the Euro-transplant Program to demonstrate the relative efficacy of the complement fixation and microcytotoxicity techniques for histocompatibility typing.

Significance to NIAID Program and Bio-Medical Research: This contractor has an excellent facility for obtaining good serum and is the principal source for reagents for the platelet complement fixation tests. This procedure makes it possible to greatly increase the number of typings done by a single technician. The platelets used in the test are readily transportable and survive for long periods of time. This overcomes the problem of perishability which exists with the cytotoxicity technique. The continued operation of this contract makes it possible to type for an increasing number of antigens by the CF technique.

Proposed Course of Project: Continuation through December 23, 1971

Date Contract Initiated: June 24, 1965

Current Annual Level: **Prior Year Support**

MASSACHUSETTS GENERAL HOSPITAL (PH43-67-1348)

Title: Evaluation of histocompatibility testing in man

Contractor's Project Director: Dr. Henry J. Winn

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor evaluated the serological leukocyte typing methods, combining them with the mixed lymphocyte culture technique to match donors and recipients; he also has studied a platelet complement fixation method as a means of tissue typing. In addition, he characterized antibodies that develop in the recipients of renal grafts and conducted a screening program for typing antisera.

Major Findings: By using both the serologic and mixed lymphocyte culture techniques to study the families of relate ddonors and recipients, a good correlation was found between HL-A haplotype identity of donors and recipients and clinical performance of the grafts. In studying recipients of renal grafts, both the presence of antibodies against donor antigens at the time of transplant and their early development were correlated with poor clinical courses and histologic evidence of vascular lesions. Valuable sera have been procured for the Serum Bank by the contractor.

Significance to NIAID Program and Bio-Medical Research: The combination of serological techniques and mixed lymphocyte culture provides a means of thorough genetic analysis and makes possible accurate matching of donor and recipient. The study of the development of antibodies against donor antigens by the graft recipients has provided a basis for graft prognosis and a possible means of anticipating rejection crises, and the accompanying vascular pathology suggests a site of action of the presumably destructive antibody-antigen reaction.

Proposed Course of Project: Terminated August 6, 1970

Date Contract Initiated: June 7, 1967

Current Annual Level: Prior Year Support

MEDICAL COLLEGE OF VIRGINIA - VIRGINIA COMMONWEALTH UNIVERSITY (PH43-68-659

Title: Analysis of antigenic and serologica factors important in human organ transplantation.

Contractor's Project Director: Dr. James C. Pierce

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is using the lymphocytotoxicity technique of leukocyte matching and the immune adherence method of kidney cell matching to compare their value in selection of donors and recipients and to determine qualitative antigenic differences in the two types of cells. He also is studying graft recipients to determine if histocompatibility antibodies may be used to predict graft rejection.

Major Findings: The contractor has constructed a model based on the current understanding of the genetics of the HL-A system, and has shown that this system can adequately account for the observed incidence of hyperacute rejection in recipients of multiple transplants. He has also demonstrated that kidney cell crossmatching can sometimes demonstrate pre-sensitization to a prospective donor in the face of a negative lymphocyte cross-match. Six patients followed closely post-transplant showed acute rises in histocompatibility antibodies at the time of rejection episodes.

Significance to NIAID Program and Bio-Medical Research: By detecting immune adherence antibodies, kidney cell cross-matching shows promise as a major improvement in histocompatibility matching. The study of the development of antibodies in post-transplant patients provides data on the immunological events occurring after transplantation, essential for understanding and modifying the rejection phenomenon.

Proposed Course of Project: Continuation through December 13, 1972

Date Contract Initiated: January 15, 1968

Current Annual Level: $94,293 (18 months)

MICROBIOLOGICAL ASSOCIATES INCORPORATED

Title: Preparation of human histocompatibility typing trays and evaluation of human histocompatibility typing trays and unknown serum

Contractor's Project Director: Robert-John Trapani, Ph.D.

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides facilities for the production of preloaded histocompatibility matching trays and for the testing of bulk and tray sera.

Major Findings: This contract has just been awarded.

Significance to NIAID Program and Bio-Medical Research: High-quality histocompatibility typing antiserum is still in very short supply. The use of preloaded trays containing the best obtainable serum makes it possible for U.S. transplanters to use identical reagents for typing of donors and recipients. This facilitates the exchange of organs and gives comparable results which may be statistically analyzed to determine the importance of histocompatibility matching. Typing serum is extremely perishable, being affected by temperature and carbon dioxide. Procedures have been developed under contract PH 43-68-619 to freeze dry serum in bulk and lambda quantities to facilitate shipping and storage. This contract will afford quality control facilities to insure that the serum which has been freeze dried has not lost its potency or specificity.

Proposed Course of Project: To terminate in one year

Date Contract Initiated: June, 1971

Current Annual Level: $39,565

UNIVERSITY OF MINNESOTA (PH43-68-1303)

Title: Evaluation of tissue typing methods in organ transplantation

Contractor's Project Director: Dr. Edmund Yunis

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor evaluated a cytotoxicity technique and the mixed culture technique in clinical histocompatibility typing, and characterized leukocyte and platelet antigenicity in patients transplanted with whole blood or leukocyte and/or platelet poor blood during hemodialysis. He also collected typing antisera for the Serum Bank.

Major Findings: Typings have been done on approximately 300 potential donors and recipients; the number of transplants performed has been too small to make clinical correlations. Work was begun on leukocyte and platelet antigenicity studies, using the platelet complement fixation technique. Several excellent typing sera have been submitted to the Serum Bank.

Significance to NIAID Program and Bio-Medical Research: The relatively small volume of transplants performed at this center has made the accumulation of data prohibitively slow; however, the serum acquisition program has been outstanding and has greatly enhanced the ability of the Serum Bank to serve clinical typers.

Proposed Course of Project: Terminated December 26, 1970

Date Contract Initiated: June 27, 1968

Current Annual Level: Prior Year Support

SCRIPPS CLINIC AND RESEARCH FOUNDATION (NIH-69-2090)

Title: Purified complement components and complement antisera

Contractor's Project Director: Dr. Hans J. Muller-Eberhard

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contract provides selected clinical investigators and medical researchers with highly purified human complement components and their respective antisera; an anaphylatoxin and its inactivator; and a unique complement component which is needed to detect soluble immune complexes.

Major Findings: During the past year this contractor has produced approximately 180 milligrams of complement protein and approximately 390 milliliters of complement component antisera. Over one-half of these products have already been distributed to 33 various clinical investigators to aid in their research on metabolic and graft rejection studies.

Significance to NIAID Program and Bio-Medical Research: Current methods of evaluating homograft rejection are not entirely satisfactory. This contract will aid in establishing another parameter for the clinician to use in accurately assessing or prognosing homograft rejection by *in vitro* tests which denote differential utilization of complement components in transplanted patients.

Proposed Course of Project: Continuation through May 22, 1972

Date Contract Initiated: May 23, 1969

Current Annual Level: $36,360

STANFORD UNIVERSITY (NIH-69-2064)

Title: Cytotoxicity assay automation

Contractor's Project Directors: Drs. Walter Bodmer and Elliot Leventhal

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is designing, building, and testing automated equipment for tissue typing, using a fluorochromatic cytotoxicity assay.

Major Findings: The fluorochromatic cytotoxicity assay has been adapted to photoelectric detection of fluorochromatic cells flowing through a small channel, with an increase in sensitivity over that attained with visual observation of the cells. An apparatus to automate sampling between an NIH serum tray and the flow system has been constructed. Comparisons of typing done by the machine and done manually are underway and preliminary results indicate the machine is suitable for routine use in daily typing activities.

Significance to NIAID Program and Bio-Medical Research: The equipment under development in this contract has demonstrated sensitivity equal to and in some tests, greater than manual use of the fluorochromatic assay. It has potential for decreasing the number of personnel necessary for running a tissue typing laboratory and for usage with other cells, such as fibroblasts, and in other test systems, such as complement fixation.

Proposed Course of Project: Continuation through June 16, 1971

Date Contract Initiated: June 17, 1969

Current Annual Level: $24,007 (six month period)

UNIVERSITY OF WASHINGTON (PH43-67-1435)

Title: Evaluation of tissue-typing techniques in clinical homotransplantation

Contractor's Project Director: Drs. E. D. Thomas and Thomas L. Marchioro

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor has been performing leukocyte typing and mixed lymphocyte cultures for a bone marrow transplantation program, with emphasis on familial typing to study the genetics of leukocyte antigens and on learning the relation of the closeness of matching by leukocyte typing with the clinical outcome of the transplanted marrow. He also has been collecting valuable typing antisera for the Serum Bank.

Major Findings: Approximately 200 potential bone marrow donors and recipients were typed during the first six months of the current contract period. In addition, all family members of the patients were typed. Thirty potential donors and 18 potential recipients have been tested in mixed lymphocyte culture. Six additional bone marrow transplants have been performed, making a total of sixteen. Graft-vs.-host disease has been found in HL-A matched, mixed lymphocyte culture negative donor-recipient pairs, indicating that the HL-A system as currently understood is not the sole factor determining the histocompatibility of bone marrow transplants. Neutrophil-typing in conjunction with the transplants is just getting underway. The serum screening program has produced several good typing antisera.

Significance to NIAID Program and Bio-Medical Research: Both the genetic data from the familial typing and the correlation of matching with clinical outcome are essential information to determine the ultimate role histocompatibility typing will have in bone-marrow transplantation. Neutrophil-typing offers an additional mode of studying antigens of as yet undetermined importance in the survival of bone-marrow grafts. The procurement of typing sera for the Serum Bank is essential to continue NIAID's typing reagent program.

Proposed Course of Project: Continuation through December 28, 1971

Date Contract Initiated: June 29, 1967

Current Annual Level: $41,081 (18 month period)

BIONETICS RESEARCH LABORATORIES, INC. (PH43-68-1327)

Title: A primate pre-clinical test center for immune study, primarily with ALS

Contractor's Project Director: Dr. Clement C. Darrow

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: The prinicpal objectives in this contract are to maintain a sub-human primate facility for the testing of human antilymphocyte serum (ALS) and to operate an immunology laboratory to conduct a battery of in vitro tests. This year, prinicpal emphasis is placed on in vivo testing of program-produced rabbit anti-human thymus serum, which is being readied for clinical trials.

Major Findings: During the past year, 31 antilymphocyte preparations were submitted to the contractor for study; these were all tested by a battery of in vitro techniques and 25 sera were selected for in vivo study. Thirty-seven Rhesus monkeys and three chimpanzees were utilized in this study; one-fourth of the preparations were shown to be capable of prolonging skin graft survival. The most effective material was rabbit antithymus serum or globulin. Comparisons of the standard three-dose and other ALS regimens were carried out; the three-dose method was shown to be safe tnd effective, and produced comparable skin graft survival to the longer dosage regimens. Skin graft studies with Rhesus and chimpanzees, using the same ALS for immunosuppression, showed good correlation of graft prolongation in both species. Tolerance induction studies were initiated in 13 Rhesus monkeys, using aggregated and non-aggregated heterologous gamma-globulin.

Significance to NIAID Program and Bio-Medical Research: ALS was used for immunosuppression in more than half of the kidney transplants performed during 1970. Few of these sera were of demonstrated efficacy. The only satisfactory means for testing ALS at the present time is the dermal allograft survival test. This contract provides for standardized procedures to conduct comparative testing of ALS preparations. Potency tests and pre-clinical pharmacology studies are also being carried out on a large batch of ALS which has been prepared. With the completion of these studies, this material will be used in a coordinated cooperative clinical evaluation of the efficacy of this immunosuppressive material.

Proposed Course of Project: Continuation through June 27, 1972 with pharmacological studies in Rhesus monkeys and chimpanzees. Additional sub-human primate testing will be utilized during the clinical trial period.

Date Contract Initiated: June 28, 1968

Current Annual Level: $207,255

JOHNS HOPKINS UNIVERSITY (PH43-66-924)

Title: Factors related to survival of bone marrow allografts in man

Contractor's Project Director: Dr. George W. Santos

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract evaluates and correlates methods of histocompatibility testing and immunosuppression with the clinical results of allogenic bone marrow grafts in man and studies the efficacy of cyclophosphamide as an immunosuppressive drug.

Major Findings: The graft vs. host phenomenon presents a major impediment to successful bone marrow transplantation. In the animal model, the contractor has developed a system for the administration of cyclophosphamide as a means of preventing and reversing rejection. Using these techniques, the contractor is now conducting human bone-marrow transplantation. He has transplanted nine patients since 1968. Methods have been developed to permit early detection of graft vs. host disease and new regimens are being studied in animals for the prophylaxis and treatment of this complication.

Significance to NIAID Program and Bio-Medical Research: Patients with far advanced hematological malignancies and those with congenital or acquired marrow deficiency states constitute a potential group for bone marrow transplants. The survivals, to date, have been rare; the development of good methods of immunosuppression and the control of infection should result in a markedly increased success rate. This contract affords a unique opportunity to study immunologic activity of the transplanted marrow cells in the graft vs. host phenomenon and to assay the relative importance of the histocompatibility antigens.

Proposed Course of Project: Continuation through December 28, 1972

Date Contract Initiated: June 29, 1966

Current Annual Level: $115,736 (18 months)

UNIVERSITY OF TEXAS, M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE (PH43-68-949)

Title: Suppression of immune responses with drugs and biological products: A search for more effective less myelotoxic immunosuppression

Contractor's Project Director: Dr. Evan M. Hersh

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides funds to evaluate new drugs and antilymphocyte serum for their immunosuppressive activity in transplant recipients and cancer patients; the contractor also develops new methodology for the evaluation of immunosuppression and studies the immune state in patients with uremia and a variety of malignant diseases.

Major Findings: The contractor has investigated a number of drugs and antilymphocyte serum which are currently being used in kidney and heart transplant patients. He has developed standardized methods for the measurement of immune responsiveness and has used these techniques to study patients prior to, during, and after therapy. A number of new anti-tumor agents are being evaluated.

Significance to NIAID Program and Bio-Medical Research: The information gained concerning the utility of new immunosuppressants has been most helpful in the management of the post-transplant patient. The standardization of methodology makes it possible for investigators to compare the utility of drugs in producing immune paralysis. The techniques will be most valuable when attempts are made to induce immune tolerance, since the aim of this procedure is to produce specific tolerance to histocompatibility antigens sparing immunologic reactivity to other antigens.

Proposed Course of Project: Continuation through December 20, 1971

Date Contract Initiated: June 21, 1968

Current Annual Level: **Prior Year Support**

AMERICAN FOUNDATION FOR BIOLOGICAL RESEARCH, BIO-MEDICAL RESEARCH INSTITUTE (PH43-68-619)

Title: Short-term maintenance of mammalian kidneys by continuous pulsatile perfusion *in vitro* and development of techniques for freeze-drying and storage of serum and preservation of lymphocytes.

Contractor's Project Directors: Mr. Vernon P. Perry, Drs. Theodore Malinin and Hector Montes de Oca

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor completed studies of the short-term perfusion of kidneys to be used in transplantation, including the development of an *in vitro* method of assessing renal function. The study of the preservation of lymphocytes was also completed. Work is ongoing in the development of methods of freeze-drying typing antisera.

Major Findings: Working primarily with rabbit kidneys undergoing pulsatile perfusion, it was found that changes with time in the renal clearance of para-amino-hippuric acid (PAH) and inulin provide a good method of assessing the damage incurred by the kidneys during short-term storage periods. Subsequently the renal arterio-venous differences in inulin and PAH proved to be equally valuable indicators of renal function during perfusion, and are much simpler to obtain than the clearance measurements.

With respect to the freeze-drying of typing sera, a 48-hour cycle has been developed to accomplish this, using both 1 ml. and 1 lambda quantities of sera, the latter on NIH trays. Shelf-life studies comparing the effect of different HL-A specificities at various titers have shown no loss of activity after six months at room temperature. Freeze-drying of a portion of the Serum Bank inventory is to begin shortly. Lymphocytes have also been frozen successfully in bulk and lambda quantities with over 90% viability after thawing.

Significance to NIAID Program and Bio-Medical Research: The difficulties in preserving kidneys and in assessing their functional status after a period of preservation have provided a major obstacle to progress in clinical transplantation. This contractor has shown that short-term preservation via perfusion with non-cellular media is possible and has provided a suitable means of assessing renal function during perfusion. It is felt that further significant progress in this field is dependent on more fundamental research in physiology and cryobiology.

The freeze-drying of typing sera will eliminate much of the expense and many of the logistical difficulties in the shipping of frozen sera and NIH typing trays, and is considered extremely important for the future operation of the Serum Bank. Lymphocyte preservation is considered highly desirable since it would enable laboratories to maintain a large panel of well-characterized cells for the testing of histocompatibility antisera.

AMERICAN FOUNDATION FOR BIOLOGICAL RESEARCH (CONTINUED)

Proposed Course of Project: Continuation of serum freeze-drying through July 31, 1971.

Date Contract Initiated: March 1, 1968

Current Annual Level: **Prior Year Support**

UNIVERSITY OF MINNESOTA (PH43-68-99)

Title: Preservation of multicellular organisms and whole organs in vitro with emphasis on the canine at sub-zero temperatures.

Contractor's Project Director: Dr. Richard C. Lillehei

Project Officers: Dr. Douglas L. Brand and Mr. C. Eugene Harris

Objectives: This contractor is developing methods of resuscitation of kidneys after freezing and storage and is studying methods of assessing cellular and organ damage caused by low temperatures.

Major Findings: Methods and equipment have been developed to perfuse kidneys with temperature-controlled fluorocarbon liquid and to rapidly thaw the organs with microwave energy, thus achieving strict control of freezing and thawing rates. Damage incurred by an organ during the freezing-thawing cycle can be assessed by the levels of two enzymes (SGOT, LDH) released into the effluent perfusate. Principles of medical treatment for frostbite are being applied in the resuscitation of frozen kidneys, and special techniques of freezing, including the use of hyperbaria along with perfusates such as glycerol, are in use to decrease damage by intra- and extra-vascular ice formation.

Significance to NIAID Program and Bio-Medical Research: The inability to preserve donated organs for more than several hours is one of the greatest obstacles to the most effective use of organs available for transplant. This contract represents an interdisciplinary approach to the task of freezing organs, bringing together the expertise of physicians, animal scientists with experience in cell freezing, a cryobiologist and mechanical engineers.

Proposed Course of Project: Continuation through February 28, 1972

Date Contract Initiated: December 29, 1967

Current Annual Level: $160,898

RESEARCH FOUNDATION OF THE STATE UNIVERSITY OF NEW YORK AT BUFFALO (PH43-67-87)

Title: Characterization of immunological phenomena accompanying homotransplantation.

Contractor's Project Director: Dr. Felix Milgrom

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor studies the immunological phenomena in humans undergoing organ transplantation in an effort to evaluate the serological and immunohistological occurrences prior to and after acute and chronic rejection of the transplanted kidney. He will ascertain the production and importance of kidney specific autoantibodies and immune complexes deposited in the renal tissue of a rejected graft in an effort to elucidate their role in the rejection process. While making these critical evaluations, he will additionally compare certain serological procedures' and reagents' ability to detect minor changes in antibody content in serum and/or antigen-complex content in the tissues.

Major Findings: This contract has been significantly productive in that it has helped to enhance our knowledge of the homograft rejection process, especially, the hyperacute rejection phenomenon, the role of autoimmunity in graft rejection, and the development of sensitive serological procedures which may detect and predict rejection of renal grafts. The developed mixed agglutination test detects antibodies in pretransplant sera of all recipients who hyperacutely rejected their renal grafts including some sera which were lymphocytotoxically negative.The preformed antibody-mediated graft rejection (hyperacute) is accompanied by moderate to dense accumulation of polymorphonuclear neutrophils in glomerular and peritubular capillaries with subsequent rapid renal cortical necrosis. A histological picture of mononuclear cell infiltration of the graft, on the other hand, indicates an acute rejection process starting about three days after the graft implant, with graft destruction occurring about ten days after transplant.

Significance to NIAID Program and Bio-Medical Research: The nephritic syndrome which developes after transplantation is a major threat to successful renal transplants. The elucidation of the role of preformed histoincompatible antibody, autoantibody and isoantibody, as well as the mechanism of cellular immunity incident to renal graft rejection is important to our overall understanding of the rejection phenomenon and its immunological implications.

Proposed Course of Project: Continuation through July 31, 1970

Date Contract Initiated: December 1, 1966

Current Annual Level: **Prior Year Support**

SCRIPPS CLINIC AND RESEARCH FOUNDATION (PH43-68-621)

Title: To identify antibodies in diseased human kidney

Contractor's Project Director: Dr. Frank J. Dixon

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor analyses human serum and renal tissue samples from transplanted patients for various antigen-antibody complexes involved in the genesis of glomerulonephritis and the etiological events which trigger the immune response leading to glomerulonephritis. This contractor will develop a more sensitive detection test for serum antiglomerular basement membrane antibodies and further evaluate these antibodies in antilymphocyte globulin (ALG) preparations currently used in the therapy of transplanted patients. Additionally, the contractor will seek microbial agents responsible for chronic human glomerulonephritis and elucidate the nature of antibodies involved in circulating immune complex nephritis.

Major Findings: This contractor has histologically and serologically evaluated 429 renal biopsy or nephrectomy specimens from transplanted kidney patients. Approximately 70 percent of the transplanted kidney specimens appear to have some degree of glomerulonephritis, immune complex nephritis, and/or anti-glomerular basement membrane nephritis. This research questions the status of graft rejection and has helped define the mechanism of renal failure in transplanted patients as being a possible recurrent nephritis rather than an immunological graft rejection. Additionally, twenty percent of a group of patients with immune complex glomerulonephritis had greatly elevated antibody titers to E B virus (Herpes-like), which may be the etiological agent of this type of nephritis.

This contractor has also evaluated ALG preparations used in the therapy of transplanted patients and has found that seventy percent of the ALS preparations produced with solid lymphoid organ lymphocytes contained glomerular-basement membrane antibodies. When tested in monkeys, one ALG preparation produced a fatal glomerulonephritis. Similarly, two human liver homograft recipients who were treated with equine ALS containing GBM reacting antibodies had equine immunoglobulin bound to their glomerular basement membrane.

Significance to NIAID Program and Bio-Medical Research: This significant research has developed evidence relative to the understanding of acute and chronic glomerulonephritis and its occurrence in transplanted kidneys. It has contributed to the elucidation of the rejection process by the addition of another parameter which must be evaluated by the clinician in management of the transplanted patient. Similarly, all ALS preparations should be screened for presence of anti-glomerular basement membrane antibodies.

Proposed Course of Project: Continuation through March 1, 1973

Date Contract Initiated: January 2, 1968

Current Annual Level: $163,398 (two years)

DUKE UNIVERSITY (NIH-71-2335)

Title: Partial support for participation in WHO histocompatibility testing workshop

Contractor's Project Director: Dr. D. Bernard Amos

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides partial support to conduct histocompatibility testing on selected populations as part of the WHO-sponsored Fifth International Conference and Workshop on Histocompatibility. Specifically, funds are provided to enable the contractor to obtain white cells from racial isolate groups in Ethiopia, Australia, and Guatemala. The cells will be kept viable by liquid nitrogen freezing and will be transported to the contractor's laboratory, where they will be tested with a battery of sera selected by the WHO Expert Committee. The results of this testing will be analyzed and will form the basis of a report which will be given at the meeting of the Committee in the fall of 1972. This study is designed to search for exotic antigens in geographically and culturally isolated groups. The discovery of these antigens may lead to a better understanding of the inconsistencies of histocompatibility matching; linkage studies wihh red cell antigens will be carried out. The contractor also hopes to determine if there is any relationship between the presence of certain histocompatibility antigens and susceptibility to tropical diseases.

Major Findings: The study is just underway; no results have been analyzed to date.

Significance to NIAID Program and Bio-Medical Research: At the present time there is good evidence that all of the histocompatibility antigens in the two segregant series of the HLA locus have not been discovered. This may be an explanation for rejection which sometimes takes place in transplants where, using presently available reagents, the donor and the recipient seem to be well matched. The discovery of missing antigens and their antisera would lead to an improved success rate for organ transplantation.

Proposed Course of Project: To terminate August 31, 1971

Date Contract Initiated: May 1, 1971

Current Annual Level: $5,856

UNIVERSITY OF CALIFORNIA, LOS ANGELES (NIH-71-2334)

Title: Partial support for participation in WHO histocompatibility testing workshop

Contractor's Project Director: Dr. Roy Walford

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides partial support to conduct histocompatibility testing on selected populations as part of the WHO-sponsored Fifth International Conference and Workshop on Histocompatibility. Specifically, funds are provided to enable the contractor to obtain white cells from racial isolate groups in Bolivia and Peru. The cells will be kept viable by liquid nitrogen freezing and will be transported to the contractor's laboratory where they will be tested with a battery of sera selected by the WHO Expert Committee. The results of this testing will be analyzed and will form the basis of a report which will be given at the meeting of the Committee in the fall of 1972. This study is designed to search for exotic antigens in geographically and culturally isolated groups. The discovery of these antigens may lead to a better understanding of the inconsistencies of histocompatibility matching, linkage studies with red cell antigens will be carried out. The contractor also hopes to determine if there is any relationship between the presence of certain histocompatibility antigens and susceptibility to tropical diseases.

Major Findings: The study is just underway; no results have been analyzed to date.

Significance to NIAID Program and Bio-Medical Research: At the present time there is good evidence that all of the histocompatibility antigens in the two segregant series of the HLA locus have not been discovered. This may be an explanation for rejection which sometimes takes place in transplants where using presently available reagents, the donor and the recipient seem to be well matched. The discovery of missing antigens and their antisera would lead to an improved success rate for organ transplantation.

Proposed Course of Project: To terminate August 31, 1971

Date Contract Initiated: May 1, 1971

Current Annual Level: $6,868

PETER BENT BRIGHAM HOSPITAL (PH43-68-1408)

Title: Operation of the kidney transplant registry

Contractor's Project Director: Dr. Joseph E. Murray

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provided funds for the operation of an international registry of kidney transplants, utilizing computerized techniques. A storage and retrieval system was established to permit statistical analysis of a number of parameters.

Major Findings: This was the terminal year of this contract. The Kidney Registry was transferred on February 11, 1971 to the American College of Surgeons/National Institutes of Health Registry, which is funded by the NIAID, NCI, NHLI, NIDR and NIAMD. This year's funding has provided for the analysis of the data to that date, the preparation of the final manuscript, and the orderly transfer of records to the American College of Surgeons Registry. Three-thousand-six-hundred and forty-five kidney transplants were reported through January 1, 1970. There was a 75% one-year kidney survival rate on transplants from consanguineous living donors and a 52% survival of kidneys transplanted from cadaver donors. This facility collects a sufficiently large sample of data on kidney transplantation, with greater validity than data obtained from smaller series. A long-time follow-up (8 - 11 years) of significant numbers of patients has also been achieved. The final report of the Peter Bent Brigham Kidney Transplant Registry was published in the March 1971 issue of Transplantation and is a compilation of all the data received from 1963 through January 1, 1970.

Significance to NIAID Program and Bio-Medical Research: This contract provided an important source for statistical information concerning the survival of kidney transplants. The census has proved extremely useful in the planning process for new Kidney Diseases programs.

Proposed Course of Project: This project was phased out on February 11, 1971. The continued operation of the registry will be through a companion contract with the American College of Surgeons which also collects information on heart, liver, and bone marrow transplants; by combining the two contracts, a single source for all experimental transplantation information has been established.

Date Contract Initiated: June 28, 1968

Current Annual Level: **Prior Year Support**

STANFORD UNIVERSITY (NIH-71-2336)

Title: Partial support for participation in WHO histocompatibility testing workshop

Contractor's Project Director: Dr. Rose Payne

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides partial support to conduct histocompatibility testing on selected populations as part of the WHO-sponsored Fifth International Conference and Workshop on Histocompatibility. Specifically, funds are provided to enable the contractor to obtain white cells from Chinese-Americans in California and Indians in New Mexico and Arizona. The cells will be kept viable by liquid nitrogen freezing and will be transported to the contractor's laboratory where they will be tested with a battery of sera selected by the WHO Expert Committee. The results of this testing will be analyzed and will form the basis of a report which will be given at the meeting of the Committee in the fall of 1972. This study is designed to search for exotic antigens in geographically and culturally isolated groups. The discovery of these antigens may lead to a better understanding of the inconsistencies of histocompatibility matching, linkage studies with red cell antigens will be carried out. The contractor also hopes to determine if there is any relationship between the presence of certain histocompatibility antigens and susceptibility to tropical diseases.

Major Findings: The study is just underway; no results have been analyzed to date.

Significance to NIAID Program and Bio-Medical Research: At the present time there is good evidence that all of the histocompatibility antigens in the two segregant series of the HLA locus have not been discovered. This may be an explanation for the rejection which sometimes takes place in transplants where, using presently available reagents, the donor and the recipient seem to be well matched. The discovery of missing antigens and their antisera would lead to an improved success rate for organ transplantation.

Proposed Course of Project: To terminate August 31, 1971

Date Contract Initiated: May 1, 1971

Current Annual Level: $3,114

NAVAL MEDICAL RESEARCH INSTITUTE, NATIONAL NAVAL MEDICAL CENTER Interagency Agreement

Title: Preparation and testing of anti-human thymocyte serum

Contractor's Project Director: Dr. Kenneth W. Sell

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides partial support for a program to immunize rabbits with human thymus cells to produce antilymphocyte serum. The product developed, following extensive pre-clinical pharmacology and toxicity studies conducted under contract PH 43-68-1327, will be used for a clinical trial.

Major Findings: Optimum methods for the production and testing of rabbit anti-human thymus serum have been developed. Pilot batch production is under way to permit sub-human primate pharmacology and toxicity studies needed for a DBS investigation and new drug application. Production of a 50-patient supply of antiserum has been started.

Significance to NIAID Program and Bio-Medical Research: This facility is producing an antilymphocyte serum of demonstrated efficacy in the prolongation of skin grafts in subhuman primates. The material is being produced under strict quality control procedures to assure potency and purity. Program studies in the past have indicated that very few of the currently employed ALS preparations can be demonstrated to have skin graft surviving properties in sub-human primates. Antilymphocyte serum is needed as an additional measure to prevent homograft rejection. This contract will produce an effective ALS which the program plans to use in a coordinated-protocol study to determine the efficacy of ALS in preventing or reversing homograft rejection.

Proposed Course of Project: To terminate December 31, 1971

Date Contract Initiated: July 1, 1970

Current Annual Level: $154,000

4. PUBLICATIONS

A

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INFECTIOUS DISEASE BRANCH
Summary

The major activities during Fiscal Year 1971 involved an in-depth review by Staff and the Infectious Disease Advisory Committee of the Respiratory Virus Vaccine Program and the establishment of a Bacterial Meningitis Vaccine Program.

In the fall of 1970, a meeting was held at National Institutes of Health to discuss recent developments in bacterial polysaccharide vaccines. Attendees included experts in polysaccharide chemistry, immunology, vaccine preparation and epidemiologists. Following two days of presentations and discussion, the Infectious Disease Advisory Committee recommended to the Director, NIAID, the establishment of a vaccine program for meningococcus and Hemophilus influenzae type b.

Meningococcal group A and C polysaccharide vaccines were evaluated in several hundred children. The vaccine induced antibody in most vaccinees, although the levels of antibody were much less in very young children than in older children and adults. A more sensitive assay utilizing radioimmuno-precipitation is under development and evaluation.

A contract was initiated for the production of a small lot of Hemophilus influenzae type b polysaccharide vaccine, clinical evaluation of this vaccine in young children, and the development of a radioimmunoassay for the detection of antibody following immunization and natural disease.

Six monovalent pneumococcal polysaccharides were produced and phase I clinical evaluation initiated in the pneumococcal vaccine program. Surveillance for pneumococcal disease in over 300,000 subjects has continued. The incidence of pneumococcal disease has been studied in various populations including health cooperatives, institutions for the aged, Navajo Indians, and other high risk groups.

A single contract for the clinical evaluation of M protein streptococcal vaccine gathered more data regarding the safety and antigenicity of this vaccine preparation. An efficacy trial of the vaccine in volunteers was initiated.

An inactivated Mycoplasma pneumoniae vaccine was produced and tested for antigenicity and safety in volunteers. The vaccine induced antibody in 95% of vaccinees. Protective efficacy of the vaccine is being tested in previously vaccinated volunteers challenged with live organisms. A large lot of vaccine is being produced for field testing this summer.

Following the licensing of rubella vaccines, the Rubella Vaccine Program was phased out. One contractor will continue to monitor vaccine efficacy, persistence of antibody and long-term safety of rubella vaccines, and also, the protection afforded by high-titered rubella gamma globulin.

In the Respiratory Virus Vaccine Development Program, live candidate respiratory syncytial virus vaccines were evaluated in young children. A temperature-sensitive mutant successfully infected children without causing disease. An inactivated parainfluenza type 3 vaccine is being prepared for subsequent intranasal and subcutaneous application. A comprehensive review of ongoing epidemiological studies demonstrated the importance of parainfluenza and respiratory syncytial viruses in causing serious lower respiratory tract illness in infants and very young children.

Adenovirus types 4 and 7 live oral vaccines were successfully employed by the military in the prevention of acute respiratory disease among recruits. Direct financial savings afforded by these vaccines are estimated at fifty to one hundred thousand dollars per recruit training center per week.

A Smallpox Vaccine Program, designed to see if a better smallpox vaccine or vaccination procedure could be devised, began to accumulate data on four different smallpox vaccines. Contractors follow a common protocol. Strain and dose of vaccine are being evaluated along with route of administration. Four academic institutions and the National Communicable Disease Center are participating in this program.

A new program on antiviral substances was well under way. Efforts are being concentrated on interferon and interferon inducers. Areas investigated include interferon standards, assay, production and purification, animal models, interferon inducer enhancement and clinical studies.

During Fiscal Year 1971, a program was instituted for the development of polysaccharide vaccines derived from Neisseria meningitidis and Hemophilus influenzae type b. Vaccine development studies were actively pursued for Streptococcus pneumoniae, Streptococcus pyogenes, Mycoplasma pneumoniae, respiratory viruses, and vaccinia. In addition, an Antiviral Substances Program became fully operational.

Meningitis Vaccines

Under the sponsorship of the NIAID, a meeting was held in the fall of 1970 on polysaccharide vaccines. This meeting was attended by eighty scientists and included many experts in this and related fields. The Infectious Disease Advisory Committee discussed the whole field of bacterial polysaccharide vaccines and recommended to the Director, NIAID that a program be initiated for the development and clinical evaluation of meningococcal and Hemophilus influenzae type b vaccines. An ad hoc committee was appointed to review the technical aspects of vaccine preparation, purification, stability and standardization, the nature of the immune response and its assay, and plans for the clinical evaluation of these vaccines in children.

A contract proposal for the development of a radioimmunoassay method for the measurement of antibody to meningococci was received, evaluated and referred to the Research Contracts Branch for implementation. A contract proposal for the preparation of Hemophilus influenzae type b vaccine, its clinical evaluation and the development of a radioimmunoassay method was similarly processed.

Hemophilus influenzae type b and meningococcus A, B and C are the principal causative agents of bacterial meningitis. Although antibiotic treatment is usually successful in the treatment of these diseases, it is disquieting to note that the mortality ranges from five to ten per cent. It has been reliably estimated that there are 25,000 cases of meningitis annually in the United States due to Hemophilus influenzae b and 10,000 cases due to meningococci. About 90% of these cases occur in children. In addition, a significant portion of very young children recover from an acute episode of meningitis with some permanent neurological deficit.

Small feasibility studies were continued by four contractors with the meningococcal group A and C polysaccharide vaccines made available by the Walter Reed Army Institute of Research. Experience in the military had provided data that both vaccines were antigenic and safe in adults, and that the group C vaccine was protective, but it was not known whether the vaccines would act as primary immunogens in young children. Approximately 300 young children were vaccinated without any untoward effects. Antibody developed to lower titer and appeared later than in adults. In infants, antibody levels were barely discernable. Thus, there is a need for a more sensitive antibody detection method, such as the radioimmunoassay.

From previous studies with agammaglobulinemic patients treated with gamma globulin, it is known that these patients are protected against meningitis even though antibody levels in their sera cannot be detected with the present methodology. Therefore, it would appear reasonable to assume that minute amounts of serum antibody in infants induced by meningococcal polysaccharide would be protective.

Pneumococcal Vaccine Program

In the fall of 1970, as part of the meeting on polysaccharide vaccines, the Infectious Disease Advisory Committee reviewed the Pneumococcal Vaccine Program. This review encompassed the entire program, including epidemiological surveillance, vaccine production, serological methodology and clinical evaluation of vaccines. Following the recommendation of the Committee, several changes of emphasis and direction by the program were effected, including stepped-up activity in vaccine production, and the strengthening of epidemiologic surveillance.

In September, 1970, the Biological Laboratories, Department of Public Health, Commonwealth of Massachusetts, began work to develop a standardized mouse protection test to measure biological potency of fourteen candidate monovalent pneumococcal vaccines. This group of investigators is experienced in bioassay methods for evaluating bacterial vaccines for immunogenicity. Pneumococcal cultures of types 1, 2, 3, 4, 5, 7, 8, 12, 14, 18 and 19 have been tested for virulence in mice. Protocols for testing vaccines, types 1, 2, 3, 7 and 8 have been developed and the ED_{50} calculated for these antigens. These studies indicate further the desirability to establish standard reference preparations of antigens and to determine comparative ratios of the protective effect of new candidate vaccines produced by commercial manufacturers.

Contracts with the University of Pennsylvania and the University of Washington have continued to provide valuable epidemiological data that delineates the incidence of pneumococcal disease in certain high risk groups. Seven defined populations, totaling almost 300,000 subjects, are included in the surveillance activity.

Analysis of surveillance data submitted from the South African Institute for Medical Research indicates that pneumococcal pneumonia is endemic and there is a high incidence of disease among laborers working at gold mines in the vicinity of Johannesburg. Pneumococci have been isolated from 76% of the cases of pneumonia. From information gathered so far, it would appear that this population with a high prevalence of disease would be a target group to consider for future field trials to determine efficacy of a polyvalent vaccine.

Through an intra-agency agreement with the Indian Health Service, epidemiological studies have shown that there is a high incidence of pneumonia of pneumococcal etiology among the Navajo Indian population of 120,000 individuals. These preliminary findings are suggestive that this would be another population suitable for field trials to demonstrate the protective efficacy of a vaccine. A contract with the University of New Mexico was awarded near the close of the fiscal year to expand field surveillance and structure the project for vaccine testing.

In addition to the contractural arrangements for surveillance, pneumococcal isolates and convalescent sera have been obtained, without funding, from over nine city hospitals throughout the country. A total of 2,000 pneumococcal infections accompanied by bacteremia were observed in these institutions over the past several years. Six pneumococcal serotypes account for 55% of the bacteremias, and twelve serotypes for 75%.

The contract with Eli Lilly and Company for the production of fourteen serotypes of pneumococcal polysaccharide has made significant progress during the past year. Twelve of fourteen antigens have been produced in bulk form. Monovalent vaccine of types 1, 2, 3, 5, 7 and 8 have been prepared, tested in the laboratory, and made available to clinical evaluation centers for antigenicity and safety testing.

Vaccine testing has been initiated in approximately 200 adults with monovalent vaccines, types 1, 2, 3, 7 and 8. Varying doses of each monovalent vaccine have been administered to adult volunteers. Pre- and post-vaccination serum specimens have been assayed for specific antibody levels by means of the radioimmunoassay and the indirect hemagglutination test. There is preliminary evidence to indicate that candidate vaccines (types 1, 2, 3, 7 and 8) are clinically safe and antigenic. As additional monovalent vaccines become avilable from Eli Lilly and Company, they will be tested in a similar manner. Monovalent vaccines will then be combined to form a polyvalent vaccine for field trial evaluation.

Investigators at the University of California, San Francisco, are developing a standardized indirect hemagglutination (IHA) test for the sero-diagnosis of pneumococcal disease. The IHA test will be further adapted to measure and differentiate antibody to at least fourteen type-specific pneumococcal polysaccharide antigens. The contractor will assume the role of a reference laboratory in evaluating the immune response to candidate monovalent and polyvalent vaccines.

Streptococci

Clinical trials in eighty-seven infants with several streptococcal M protein vaccines of five separate serotypes have demonstrated that the vaccine induces serum antibody. Almost all children responded with type-specific hemagglutinating antibody, but only about one-half developed bactericidal antibody. The magnitude of the antibody response was influenced by the nature of the immunizing antigen. Potency of vaccines in rabbits correlated very well with the human response.

Sera from rabbits hyperimmunized with vaccine and sera from vaccinated infants did not contain heart-reacting antibodies.

Adult volunteers have been immunized with vaccine in preparation for a challenge study to determine protective efficacy. If this and subsequent studies prove successful, efforts will be made to expand this study from the present one contractor to include additional vaccine evaluation centers.

Mycoplasma pneumoniae

Through a collaborative effort of three contractors, an inactivated *Mycoplasma pneumoniae* vaccine was developed and prepared. This vaccine was 95 - 100% antigenic in a clinical evaluation study conducted among adult volunteers by three other contractors. A study to prove the protective efficacy of the vaccine in a challenge situation is now under way. Two large lots of vaccine have been made in preparation for a field trial in summer of 1971. Furthermore, the vaccine will be evaluated for antigenicity in children.

Respiratory Virus Vaccine Development Program

In January and March 1971, Staff, contractors and the Infectious Disease Advisory Committee reviewed in depth the Respiratory Virus Vaccine Program. Topics discussed included epidemiological studies, serological methodology, vaccine development and clinical evaluation. Top priority was recommended for respiratory syncytial virus and parainfluenza viruses, intermediate priority was accorded the adenoviruses and low priority for rhinoviruses.

Respiratory Syncytial Virus - Parainfluenza Viruses

A review of ongoing epidemiological studies by the Advisory Committee served to emphasize the finding that RS and parainfluenza viruses are the principal causes of severe lower respiratory tract disease among infants and children. The need for effective vaccines to reduce the morbidity and economic toll of these viruses in infants was apparent. A low-temperature adapted RS virus vaccine induced a silent infection in children, but on testing with young infants, was associated with respiratory disease. A temperature-sensitive (Ts) mutant, developed by the Laboratory of Infectious Diseases, NIAID, was shown to be more attenuated than the low-temperature adapted strain. This Ts mutant has now been evaluated in children and in a few infants, all of whom had pre-existing antibody as a result of prior natural infection. The mutant effected colonization of the upper respiratory tract without inducing illness. The mutant will be given to a small number of young infants without pre-existing antibody in summer of 1971.

Parainfluenza viruses of type 1, 2 and 3 grown in eggs at the 30th, 60th and 80th passage level were clinically evaluated for infectivity in adult volunteers. All three vaccines failed to induce colonization of the upper respiratory tract and none induced antibody formation. A concentrated inactivated parainfluenza type 3 vaccine is being produced for intranasal immunization, and an inactivated parainfluenza type 1 vaccine is scheduled for clinical evaluation. In epidemiological and vaccine evaluation studies, secretory antibodies as well as serum antibodies are being assayed to better define their relative roles in affording protection against disease.

Adenoviruses

The simultaneous administration of live oral adenovirus type 4 and 7 vaccines was undertaken in extensive field trials by the Armed Forces. The two vaccines together were highly effective in the control of acute respiratory disease among military recruits. As a result of their success during the 1970-71 respiratory season, these vaccines have been adopted for routine use by the military and therefore, this aspect of the Adenovirus Program was terminated. Live oral adenovirus type 1, 2, 5, 14 and 21 vaccines have been clinically evaluated in adult volunteers. Each of these five vaccines induces a silent infection of the gastrointestinal tract with the development of serum antibody. Adenovirus type 1, 2 and 5 vaccines are now scheduled for evaluation in children, the target population for these "pediatric" adenovirus types.

In addition to live oral adenovirus vaccines, an inactivated DNA-free soluble antigen vaccine for type 5 adenovirus has been developed by National Drug Company. Clinical evaluation is just getting under way. A crystalline adenovirus type 5 hexon was prepared through a collaborative effort of Dr. Julius Kasel, Laboratory of Clinical Investigation, NIAID and Dr. Pereira, Mill Hill, England. The crystalline vaccine is now being evaluated in adult volunteers. If either the soluble antigen vaccine or the crystallized hexon is immunogenic, then the final goal of a truly purified adenovirus vaccine will have been achieved.

Rhinoviruses

Eighty-nine distinct serological types and one subtype of rhinoviruses have been established by a collaborative effort of contractors participating in the rhinovirus typing program. Several candidate rhinovirus prototypes have been shown to possess one-way cross-reactions with established prototypes. The use of combinatorial antiserum pools has simplified and accelerated the typing of rhinovirus isolates, and aided in the selection of candidate rhinovirus strains for inclusion in phase III of the rhinovirus typing program.

With the exception of military bases and possibly certain other small defined populations, there is no preponderance of a limited number of rhinovirus serotypes in association with respiratory disease. Although stimulating serum antibody, inactivated rhinovirus vaccines have been ineffective in conferring protection in clinical studies. Live rhinovirus vaccines have been quite variable in efficacy trials among volunteers. The combination of a multiplicity of serotypes, lack of a limited number of serotypes in association with disease, absence of protective efficacy for inactivated vaccines, and generally poor response with live vaccines as well as the relatively minor nature of rhinovirus disease make prospects for the successful development and utilization of rhinovirus vaccines rather unlikely. With the exception of the development of a feasible rhinovirus vaccine, the original aims of the program - epidemiology, typing, and immunology - have been successfully achieved, and therefore, the program is being phased out.

Rubella

With the exception of one longitudinal study of rubella vaccine efficacy and safety, the Rubella Vaccine Program has been phased out. There is still a need for good data on the effects of rubella vaccine inadvertently administered to pregnant women. It is now known that vaccine virus can infect the placenta, and may infect the embryo itself, although it still remains to be demonstrated whether the vaccine strain is teratogenic.

Children vaccinated with various rubella vaccines are being followed for persistence of antibody and resistance to infection and disease. Only moderate protection has been demonstrated with high-titered rubella immune globulin.

Smallpox

Because of the increasing concern regarding the more serious complications which arise from the use of the current smallpox vaccine, the NIAID decided to initiate a collaborative study to select a better smallpox vaccine or vaccination procedure.

Contracts were awarded to four investigators at four separate academic institutions for studies of four different smallpox vaccines administered by either subcutaneous or percutaneous routes and in varying concentration. The objective of the study is to determine the best method for smallpox immunization with a view to reducing, thereby, the frequency of complications which now follow conventional, non-quantitative vaccination procedures.

A total of 2400 to 2800 children will be immunized in this study. During the first eight months of these contracts, 654 children have been immunized by the percutaneous route. Standardized follow-up studies including revaccination are in progress. Only thirty children have been immunized by the subcutaneous route, because the first lots of subcutaneous vaccine dropped in titer. New lots of subcutaneous vaccine which appear to be stable have been prepared. They were diluted in water rather than phosphate buffered saline. It is thought that this collaborative study will be completed in December 1972.

While such studies would not of themselves be expected to provide any data on the more serious complications, such as post-vaccinial encephalitis, they should provide data which might enable selection of an improved vaccination procedure. A method which would carry less risk of some of the complications encountered with present vaccines and methods of vaccination would be particularly applicable to those countries now free of smallpox.

Antiviral Substances Program

There are many viral diseases for which there is no prophylaxis or therapy presently available and which constitute serious public health problems. There is, therefore, a need for the development of antiviral substances for the treatment of these diseases. Planning for a program to help meet this need began in July 1969. A thorough study of the field revealed that there was considerable increasing interest in this area and that interferon or interferon inducers were the most promising antivirals to exploit.

The developments and new information emanating from scientist-originated basic research had evolved to the point where support in some specific areas was needed. Initial emphasis by the program was concentrated in the field of interferon research. The general intent of the program is to serve as a focus for all activities on antivirals conducted by Extramural, Intramural and Collaborative Research of the NIAID, and to identify and support research in the gap areas which need further support so as to hasten the field in applying these compounds to man.

Several ad hoc committees were assembled to discuss the field and more specifically, the areas wherein a contract program would be most appropriate in supporting the ongoing research for application of this information to health care. Several areas were suggested for development and contracts were initiated in spring, 1970, in some of these areas. They included:

Assay: In order to support and expedite research in the area of interferon, studies were undertaken to develop a more rapid, quantitative and standardized assay. Work was initiated to examine a variety of parameters to achieve this goal. One assay system using human amnion U cells and VSV has reduced the time from 72 to 24 hours per assay. Another new assay based on the reduction in yield of viral neuraminidase has been developed and this method has promise of being highly precise, rapid and economical.

Standards: In an effort to better coordinate results from the different laboratories engaged in interferon research, this program has actively participated in the establishment of international interferon and interferon-inducer standards. The program is responsible for rabbit and mouse interferon and the inducer polyinosinic:polycytidylic acid standards. It is also engaged in studies on stabilization of lyophilized interferon. When standards are established they are submitted to the NIAID Research Resources Branch for distribution.

Production: If interferon proves effective in the treatment of human viral diseases, two methods of administration are available: the administration of exogenous interferon, produced in vitro, or endogenous interferon through the use of inducers. For the former, there is a need for the development of a technique which will provide a more efficient, safe and economically feasible method of producing large quantities of human interferon. Contracts are in effect to provide the most promising cell for this production, which preliminary studies indicate is the human amnion, and the best method of interferon induction. Through this research, yields have been increased fivefold. However, greater increases are needed to render it an economically feasible method of treatment, therefore, further work is needed. A new contract is being established to provide the mechanism for large scale production, once the best cell and induction system are defined.

Purification: If exogenous interferon is to be used in man there is a need for purification and concentration. Work is being pursued in this area, the ultimate desirable goal of this study would be purification, chemical structure definition and synthetic synthesis.

Animal Models: In continued efforts to meet the urgent need of standardization in this field, several animal-models have been established. These are models in which the disease closely mimics a viral disease in man. Baseline data are now being established using exogenous interferon and the inducer poly I:poly C. This will then afford a means of testing new antivirals, as they become available, for a comparison of their relative merits. Work under contract has established that the cat and the pig do respond to interferon inducers and that prophylaxis and therapy is possible in dermatological viral infections and rabies in the rabbit. There is also some indication that therapy is possible in encephalitis using the mouse model. Other drugs being tested include rifampicin and tilorone HCl.

Clinical Studies: The program has also arranged for the purchase of a large batch of the interferon inducer, polyinosinic:polycytidylic acid so that all investigators using this compound will be working with the same material, avoiding variations which would be encountered in preparation of multiple lots. This inducer has been prepared in a manner appropriate for use in clinical studies. A portion of the poly I and the poly C has been set aside for annealing at a later date following results of a study for the best method of combining the two polymers to achieve the most effective product. Clinical trials are being contemplated using this compound in life-threatening diseases where no therapy is available. Topical application of this compound has been found to be non-toxic, therefore, a clinical trial is currently under way for the evaluation of poly I:poly C as a therapeutic agent for the treatment of herpetic keratoconjunctivitis and adenovirus keratoconjunctivitis.

New contracts initiated in June 1971 include studies on enhancing the effect of interferon inducers, reducing their toxicity and overcoming the hyporesponsive period observed following interferon induction. Research sponsored by this program will continue to be undertaken in an effort to bridge the gap from scientist-originated research to practical use in man.

ABBOTT LABORATORIES - (NIH-70-2127)

Title: Production of Crude and Partially Purified Human Interferon

Contractor's Project Director: Morton C. Bartlett, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Produce 100 million international units of human interferon.

Major Findings: The contractor was able after some difficulty to produce exogenous interferon in human embryonic kidney tissue culture. They were able to delete the serum from the media as requested, however, they were unable to get the yields they had anticipated. Since their top yield was 600 units of interferon/ml of fluid, it was determined that this was not a feasible method of producing interferon and the contract was therefore terminated.

Significance to NIAID Programs & Bio-Medical Research: This contract was to produce 100 million units of interferon in human embryonic kidney cells. This crude interferon was to be supplied to another contractor who is doing purification studies. These studies in turn are necessary if exogenous interferon is to be used in man.

Proposed Course of Project: Terminated February 21, 1971.

Date Contract Initiated: May 22, 1970

Current Annual Level: $12,000

BAYLOR UNIVERSITY - (PH43-68-963)

Title: Evaluation of Experimental Respiratory Virus Prophylactic and Therapeutic Agents in Normal Volunteers

Contractor's Project Director: Robert B. Couch, M.D.

Project Officer: Julius Kasel, Ph. D.

Objectives: To test experimental respiratory virus vaccines and chemoprophylactic agents, and to develop and evaluate principles for development of such agents.

Major Findings: Three passages in WI-38 tissue cultures were found to have resulted in attenuation of rhinovirus Type 15 as exhibited by a 20-fold increase in the HID_{50} as compared to second passage material and a marked reduction in the occurrence of illness among infected volunteers. Serum and nasal secretion antibody responses among volunteers who received second passage and third passage virus were approximately equal.

Two plaque sizes were selected from third passage material and subsequently passaged, grown up into volunteer inoculum pools, and administered to other volunteers. Although infection occurred in all challenged individuals, there was a significant difference in the occurrence of illness associated with the two plaque strains.

Type 13 and Type 1A rhinoviruses have been similarly passaged in WI-38 cells. Type 1A was tested for attenuation at the 4th, 6th, and 10th passage level and Type 13 at the 4th passage level. There was little or no evidence of attenuation, thus suggesting that tissue culture passage per se does not result in attenuation of rhinoviruses.

The National Drug parainfluenza type 3 live virus preparation at the 80th level in chick embryoes was administered nasopharyngeally to 11 individuals with low serum antibody titers, 10 of whom lacked detectable nasal secretion antibody. The challange dose was 32,000 $TCID_{50}$. None of the individuals in the followup shed Parainfluenza Type 3, or developed a serum or nasal secretion antibody response to this virus.

Mycoplasma pneumoniae inactivated vaccine, Lot 006004 - A single parenteral administration of vaccine was given to 45 volunteers. A significant frequency of brief transient soreness at the injection site occurred following vaccination. One hundred per cent of antibody-free individuals and 80% of individuals with some pre-existing antibody developed 4-fold or greater rises in growth inhibiting antibody. Additional volunteers have been immunized in preparation for a challenge study to measure the protective efficacy of the vaccine.

Significance to NIAID Programs and Bio-Medical Research: This group possesses the experience and facilities to carry out both open and closed clinical studies which are necessary for the evaluation of various vaccines in the Program.

Proposed Course of Project: This contract will be in effect until December 19, 1971. Renewal is anticipated.

Date Contract Initiated: June 20, 1968

Current Annual Level: Prior year support

UNIVERSITY OF CALIFORNIA - DAVIS - (NIH-70-2171)

Title: Production of Interferon in Cultures of Human Cells

Contractor's Project Director: Robert S. Chang, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Develop a technique for a more efficient, safe and economically feasible production of human interferon. This tissue culture expert will study a variety of human cell systems which have potential for interferon production. He will also investigate the differences between spinner and stationary cultures as well as methods of increasing cell yield.

Major Findings: The contractor has screened a number of human cells to date with the conclusion that lymphoid cells are no better than fibroblasts and that the human amnion seems to be the most interesting tissue. He has studied a number of variables in cell culture with no major enhancing effect. He is currently examining the microcarrier system to increase yield of cells and therefore interferon per ml of fluid.

Significance to NIAID Programs & Bio-Medical Research: All the interferon inducers currently available are toxic, therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 23, 1970

Current Annual Level: $28,000

UNIVERSITY OF CALIFORNIA - SAN DIEGO - (NIH-70-2263)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: James D. Connor, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A study to evaluate the reactivity and immunogenicity of four vaccinia viruses when administered percutaneously and subcutaneously for primary vaccination in infants and children was initiated during the past eight (8) months of the first contract year. Approximately 100 children have been vaccinated by the percutaneous route and 82 have been seen in follow-up. Of these, 69 have complete clinical data; 29 showed no reaction to vaccination; and 40 developed a primary "take". Of 29 without clinical response to vaccination, 11 had hemagglutinating-inhibiting antibody in post-vaccination serum; 14 no antibody; and on 4 no blood was obtained. Of 40 patients with a clinical "take" 34 had antibody in post-vaccination serum; one had no antibody; and on 5 no blood was obtained. These preliminary results show a definite correlation between a clinical "take" after primary vaccination and HI antibody response. Since no pre-vaccination sera were taken the presence of antibody in post-vaccination sera of those showing no clinical "take" may or may not be related to vaccination.

Significance to NIAID Programs & Bio-Medical Research: There is currently much concern with the smallpox vaccine due to the infrequent resulting complications. There are some who advocate abolishing compulsory vaccination. This would result in possibly greater problems. A more practical solution would be to get a better vaccination procedure. The Infectious Disease Branch has initiated this study as an immediate step toward this goal.

Proposed Course of Project: Contract has been renewed until December 21, 1972.

Date Contract Initiated: June 22, 1970

Current Annual Level: $50,000

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO - (NIH-71-2203)

Title: An Indirect Hemagglutination Test to Measure Antibody Response to Pneumococcal Polysaccharides

Contractor's Project Director: Arthur J. Ammann, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: (1) Develop a standardized indirect hemagglutination (IHA) test for a mass screening operation to measure serum antibody to pneumococcal polysaccharides following natural infection and specific immunization; (2) The serological test should be applicable to determining serum antibodies to any and all of the following serotypes of Streptococcus pneumoniae (types 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 14, 18, 19, and 23); (3) Serve as a reference serological laboratory.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs & Bio-Medical Research: There is a need for improved serological tests to improve the diagnosis of pneumococcal disease and assess the immune response in individuals receiving new experimental pneumococcal vaccine. Initiating a contract for the development of a standardized IHA is a means of implementing this specific requirement of the pneumococcal program.

Proposed Course of Project: Before the first aniversary date, progress made under this contract will be reviewed and evaluated for new direction and continuation for a second 12 month renewal period.

Date Contract Initiated: June 1971

Current Annual Level: $29,000

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO - (NIH-71-2195)

Title: Stabilization of Polyinosinic·polycytidylic Acid Against Serum Hydrolytic Enzymes.

Contractor's Project Director: R. Bhatnagar, Ph. D.
I. Zipkin, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Complexing polyinosinic·polycytidylic acid with various polyanious and non-antigenic proteins to render the interferon inducer more resistant to human serum hydrolytic enzymes. Ultimate goal is to render the inducer more effective and less toxic for use in man.

Major Findings: Since this is a new contract no data are reported

Significance to NIAID Programs & Bio-Medical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available exogenous, prepared in vitro, and endogenous through use of inducers. The inducers available are somewhat toxic including the most promising, poly I·poly C. This contract is an attempt to stabilize the compound so that the dosage can be reduced without affecting or increasing its inducing abilities.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 1971

Current Annual Level: $39,000

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH - (PH-43-68-694)

Title: Studies of Potential Respiratory Vaccine Strains and Isolation Methods

Contractor's Project Director: Robert Magoffin, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: 1) Improve and evaluate various laboratory diagnostic methods for respiratory disease surveillance and vaccine evaluation; 2) develop, select and evaluate new mutant strains of parainfluenza viruses with characteristics suitable for use as potential vaccine strains.

Major Findings: An absolute requirement for work with conditional lethal mutants is an accurate and reliable plaque assay. Plaque formation by parainfluenza virus type 3 was enhanced in the presence of DEAE dextran and magnesium chloride. Two to five-fold higher plaque counts and an increase in plaque size occurred in cultures with both additives incorporated into the overlay medium. No plaques were observed in cultures without the additives. A direct linear relationship was found between plaque count and relative virus concentration. Maximum plaque counts were obtained approximately ten days post-infection. Plaque formation occurred in the temperature range of 32° to 38°C, although significantly lower plaque counts were recorded at both 32° and 38°C. Highest plaque counts usually occurred at 37°C although no significant difference could be demonstrated in the temperature range of 33° to 37.5°C.

Two classes of conditional lethal mutants, (a) ts mutants and (b) host-dependent mutants, have been selected for in-virus stocks grown in the presence of 5-fluorouracil. Approximately 170 well-isolated plaques have been picked from mutagenized virus. Of these, 99 have been screened for both ts and host-dependent mutants. None of the virus clones tested demonstrated a sufficiently reduced capacity to form plaques at the restrictive temperature to be considered ts mutants. However, six of the plaques examined have shown properties consistent with those of host-dependent mutants. Characterization of these mutants is in progress.

Significance to NIAID Program and Bio-Medical Research: Because of the large amount of lower respiratory tract disease in infants caused by the parainfluenza viruses, there is a need for live attenuated parainfluenza vaccines.

Proposed Course of Project: To continue the development, selection and evaluation of new potential vaccine strains.

Date Contract Initiated: June 27, 1963 -- New Contract Number: PH43-68-694.

Current Annual Level: $36,000.

UNIVERSITY OF CHICAGO - (PH-43-68-83)

Title: Immunization of Humans with Group A Streptococcal M Proteins

Contractor's Project Director: Albert Dorfman, M.D., Ph.D.

Project Officer: Roger Cole, M.D.

Objectives: 1) Obtain and challenge a panel of immunized and non-immunized volunteers with type 1 streptococci. 2) Test bivalent or trivalent M preparations for immunogenicity; extend monovalent testing of M of types 1, 3 and 6; and add types 4 and 5. 3) Test booster effect of M in the previously immunized infants, in whom the levels of bactericidal antibody have disappeared.

Major Findings: During the first three years of this contract, a total of eighty-seven infants received primary subcutaneous immunizations with monovalent purified M protein vaccines of the following streptococcal serotypes: type 12, fifty-seven infants; type 6, fourteen infants; type 1, six infants; type 3, six infants; and type 14, four infants. Sera from immunized infants were tested for type-specific hemagglutinating and bactericidal serum antibodies, and the infants themselves were tested for delayed cutaneous hypersensitivity to M protein of the homologous serotype. A majority of subjects who received three injections of type 12 alum-precipitated M protein vaccine exhibited a primary and secondary antibody response in which type-specific bactericidal activity could be measured. Antibody response in rabbits correlated very well with antibody response in humans. The type 12 vaccine lots tested thus far have elicited a mild delayed cutaneous hypersensitivity to the injected antigen following three injections in infants. Other types have not been tested for skin reactivity. No systemic reactions have been observed.

Sera from vaccinated rabbits and 150 acute rheumatic fever sera were surveyed for heart-reacting antibodies. Neither the hyperimmune rabbit sera nor the human rheumatic fever sera contained heart-reactive antibodies attributable to M protein antibodies.

Bactericidal activity in saliva and nasal washings of persons with anti-M type 12 bactericidal antibodies in serum was found in 9/35 rheumatics, but in none of five adults immunized intranasally with soluble M 12.

Post-immunization sera from fifty-eight infants, anti-M hyperimmune rabbit sera, and sera from ninety-seven rheumatic fever patients were assayed for cytotoxic antibodies against human transplantation antigens. No cytotoxic antibodies were found for human transplantation antigens (HL-A) in any of the sera.

Significance to NIAID Program and Bio-Medical Research: Streptococcal disease and its sequelae i.e. rheumatic fever and glomerulonephritis are significant health problems. An effective safe vaccine would be of great importance.

Proposed Course of Project: This contract will continue until January 22, 1972.

Date Contract Initiated: January 22, 1968.

Current Annual Level: $61,000.

CHILDREN'S HOSPITAL RESEARCH FOUNDATION, COLUMBUS, OHIO - (NIH-69-2062)

Title: Rhinovirus Reference Center

Contractor's Project Director: Vincent V. Hamparian, Ph.D.

Project Officer: Albert Kapikian, M.D.

Objectives: To compare by serological methods suitable candidate prototype rhinoviruses received from collaborating laboratories throughout the world in order to define the number of prevalent serotypes and to allow an agreement to be reached by interested workers on a final numbering system.

Major Findings: Phase II of the collaborative rhinovirus program has been completed. Of seventy-three candidate prototype rhinoviruses submitted, thirty-four have been designated as new serotypes. The numbering system has now been expanded to include a total of eighty-nine rhinoviruses.

Because of one-way or low grade cross-reactions, the status of ten phase II viruses remains unresolved. The viruses involved (including five phase I viruses) have been plaque-purified again and new antisera are being prepared.

In the past year, antisera satisfactory for use as interim reagents have been prepared for thirty-four rhinoviruses. Thus, sera for all numbered rhinoviruses are available on a limited basis to qualified investigators.

A survey of the incidence of rhinovirus antibody in adults was completed. Adults appear to possess serum antibody for about 40-50% of known rhinoviruses.

A new micro-neutralization test for use with rhinoviruses has been developed.

Studies on RNA and protein synthesis in rhinovirus infected cells indicate that the accumulation of label into host RNA is inhibited at five hours after infection. The accumulation of label into host protein was inhibited at seven to eight hours. Actinomycin D did not affect protein synthesis.

Significance to NIAID Program and Bio-Medical Research: Rhinoviruses are the major cause of the "common cold". In order to find prophylactic methods for combating the "common cold" it is necessary to know the causative agent.

Proposed Course of Project: To continue through March 31, 1972.

Date Contract Initiated: May 1, 1969.

Current Annual Level: $83,000.

UNIVERSITY OF COLORADO - (PH-43-62-477)

Title: Respiratory Virus Vaccine Evaluation and Surveillance

Contractor's Project Director: Gordon Meiklejohn, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To clinically evaluate in selected populations the following vaccines: live respiratory syncytial virus, meningococcal and *Hemophilus influenzae* type b polysaccharides, and inactivated parainfluenza virus.

Major Findings: Three trials of live 26°-adapted RSV-A2 vaccine were conducted among asthmatic children at National Jewish Hospital. The vaccine was infective in nineteen of twenty-eight children and stimulated the formation of serum CF antibodies in eleven out of twenty-eight. Although there was considerable intercurrent illness in the third trial, definite pathogenicity of the vaccine was not demonstrated. The children remaining at NJH are being followed closely to evaluate protective effect.

A trial of meningococcal polysaccharide vaccines, groups A and C, was begun at the Colorado State Home and Training School. Twenty-seven children, ages four to twelve were given a single subcutaneous dose of group A vaccine, twenty-six were given group C vaccine, and twenty-four were given combined group A and C vaccine. Twenty children served as controls. Preliminary indirect hemagglutination tests indicate that the vaccines were antigenic in about 94% of children.

During 1968-69, twenty asthmatic children, ages one to five, were studied over a six-month period for respiratory virus infections. RS virus was found to be a virulent organism in this population, producing infection in eighteen children, wheezing attacks in fifteen, and pulmonary infiltrates in eleven. Coronaviruses in the OC38-43 group caused twenty-four infections, of which fourteen were associated with wheezing illness and seven with pulmonary infiltrates.

Significance to NIAID Programs and Bio-Medical Research: Provides important pediatric and adult populations for vaccine evaluation.

Proposed Course of Project: This contract will continue until June 19, 1972.

Date Contract Initiated: June 1, 1962.

Current Annual Level: $71,000.

UNIVERSITY OF COLORADO - (NIH-70-2264)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: C. Henry Kempe, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: Work has begun in the testing of 4 smallpox vaccines in various dilutions by percutaneous and subcutaneous routes. One hundred and seventy-two children have been given coded percutaneous vaccine with successful observations and 28-day serologic sampling of about 95%. Laboratory studies of HI and neutralizing antibodies are in progress.

Significance to NIAID Programs & Bio-Medical Research: There is currently much concern with the smallpox vaccine due to the infrequent resulting complications. There are some who advocate abolishing compulsory vaccination. This would result in possibly greater problems. A more practical solution would be to get a better vaccination procedure. The Infectious Disease Branch has initiated this study as an immediate step toward this goal.

Proposed Course of Project: Contract has been renewed until December 14, 1972.

Date Contract Initiated: June 15, 1970

Current Annual Level: $39,000

CORNELL UNIVERSITY MEDICAL COLLEGE - (NIH-70-2065)

Title: Study of the Antigenicity and Immunogenicity of Mycoplasma pneumoniae Vaccines and Development of an Animal Model System for the Study of Respiratory Syncytial Virus

Contractor's Project Director: Laurence Senterfit, Sc. D.

Project Officer: Robert Purcell, M.D.

Objectives: (1) To support Mycoplasma pneumoniae vaccine production at Huntingdon Research Center (HRC) and to conduct the serologic testing of sera from clinical trials with HRC vaccines. (2) To study the immunogenicity and antigenic structure of temperature sensitive (Ts) mutants of Mycoplasma pneumoniae. (3) To complete the development of methods for the qualitative and quantitative measurement of antibody to specific determinants on the mycoplasma membrane. (4) To complete the study of the "paradoxical effect" of vaccination with inactivated respiratory syncytial virus. (5) To study the adjuvant effect of polynucleotides on viral, mycoplasmal, and bacterial vaccines.

Major Findings: An experimental Mycoplasma pneumoniae vaccine produced at Huntingdon Research Center under the supervision of Dr. Senterfit and Dr. Somerson produced 100% serologic conversion in human volunteers who were seronegative prior to injection. This vaccine differed from the previous two lots produced at Huntingdon Research Center (HRC) in that (1) it was prepared from a low passage mycoplasma seed (2) the seed was from a new isolate which had never been grown on medium containing horse serum and (3) considerably more antigenic mass was present in the vaccine.

The adjuvanting effects of Poly I:C in mycoplasma and respiratory syncytial virus vaccines has been investigated. Addition of this material to both mycoplasma and respiratory syncytial virus vaccines produces a marked adjuvanting effect. No adverse sensitization effect has been demonstrated with either parenterally or intranasally administered vaccines.

In cooperation with Dr. M. Green of St. Louis University, respiratory syncytial virus has been separated from media components and cells by various physical methods. The viral material has been separated into two component antigens and antisera have been prepared in rabbits to each of the antigens. Hamsters have been injected with these purified materials and studies are in progress to determine whether there is an enhancement of the pathogenic effect of RS in animals previously injected with either of the separate antigens.

Significance to NIAID Program & Bio-Medical Research: There is a need for a better animal model system in which the serological response to potential inactivated M. pneumoniae vaccines will more closely parallel the human response. Such an animal will aid in the selection of a more potent mycoplasma vaccine.

Proposed Course of Project: This contract has been renewed until January 17, 1972.

Date Contract Initiated: January 18, 1970

Current Annual Level: $51,000

UNIVERSITY OF FLORIDA COLLEGE OF MEDICINE - (NIH-70-2170)

Title: Production of Human Interferon

Contractor's Project Director: George E. Gifford, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Develop a technique for a more efficient, safe and economically feasible production of human interferon. This contractor will investigate the potential of human tissues and the role of proteolytic enzymes in interferon production and inactivation.

Major Findings: The contractor has screened a variety of human cell types under several culture conditions and infected with various viruses at a number of different multiplicities for the ability to produce interferon. Emphasis has been placed on cells and tissues derived from the products of human conception. To date, the best system has been amnion cultures (aged over two weeks) infected with a lentogenic strain of Newcastle disease virus. This system yields 250 times more interferon than an equivalent number of lymphocytes. Attempts are being made to realize this amount of interferon directly from amnion fragments since this would simplify and decrease the cost of producing human interferon.

Significance to NIAID Programs & Bio-Medical Research: All the interferon inducers currently available are toxic, therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 23, 1970

Current Annual Level: $31,000

GEORGETOWN UNIVERSITY - (PH-43-67- 745)

Title: Studies of Local Antibody in Respiratory Viral Immunity

Contractor's Project Director: Joseph Bellanti, M.D.

Project Officers: Robert Chanock, M.D. and Daniel I. Mullally, M.D.

Objectives: 1) To complete studies of humoral and local antibody in children and adults naturally or experimentally infected with respiratory syncytial virus (RSV). 2) To complete the study of M. pneumoniae antibody in serum and nasal secretions using a radioactively labeled phospholipid hapten. 3) To develop a more sensitive test for the presence of nasal antibody to influenza virus in secretions, using a radioactively labeled influenza virus preparation.

Major Findings: Studies of serum and local antibody responses following infection with Mycoplasma pneumoniae indicate that the predominant antibody in serum is of the gamma G variety. Antibody in respiratory secretions can not be regularly detected using conventional techniques. Early results indicate that the use of radio-binding employing a radioactive C^{14} glycolipid hapten appears feasible for the measurement of antibody in situations where greater sensitivity is needed.

Studies of respiratory syncytial virus antibody in nasal secretions and sera employing radioimmunodiffusion techniques utilizing C^{14} labeled RS virus have continued. The work includes the identification of gamma E neutralizing activity in nasal secretions by selective absorption employing specific anti-gamma E antibody.

Significance to NIAID Programs and Bio-Medical Research: Investigations in this program have produced some very promising leads that local antibody (IgA) in respiratory secretions is of paramount importance in the development of immunity to respiratory illness. Volunteers receiving inactivated vaccines parenterally do not usually develop nasal antibody and can, therefore, be infected. Patients with natural infection for the most part develop nasal antibody in addition to serum antibody. These observations suggest that the ability to express antibody in nasal secretions might be an important consideration in devising methods of immunoprophylaxis against respiratory disease.

Proposed Course of Project: Contract has been renewed until April 18, 1972.

Date Contract Initiated: April 19, 1967.

Current Annual Level: $26,000.

GEORGETOWN UNIVERSITY MEDICAL SCHOOL - (NIH-70-2186)

Title: Polyinosinic acid-polycytidylic acid complexes: Studies on the variables in the formation of stable and biologically active complexes.

Contractor's Project Director: T. Screevalsan, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Study the variables in the procedure for formulating the double stranded polyinosinic·polycytidylic (In·Cn) acid in order to obtain a stable, reliable, potent and less toxic preparation of In·Cn.

Major Findings: Various studies were undertaken in an attempt to avoid the extreme variability in antiviral activity seen in the various cell lines using a single preparation of poly I·poly C. These studies suggest that the physiology of the cells in culture may play a role in the susceptibility of the cell to the poly I·poly C rather than the physical state of the compound. This study has however, devised a formulation process to obtain a more active compound. It was determined that the sequence of the addition of sodium chloride to the poly I and poly C solutions was crutial. The optimal temperature and time for hybridization was found to be 44°C and 30 minutes. Other factors studied and optimum conditions determined included phosphate concentration, pH, and melting points.

Significance to NIAID Programs & Bio-Medical Research: The current method of formulating In·Cn although adequate does not consistently result in a reliable product. There is a need for a method which would yield a consistent and less toxic In·Cn.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 26, 1970

Current Annual Level: $52,000

HEKTOEN INSTITUTE FOR MEDICAL RESEARCH OF THE COOK COUNTY HOSPITAL - (PH43-63-76; NIH-69-2092)

Title: Epidemiological Studies of Viral Respiratory Infections in Children and Vaccine Evaluation

Contractor's Project Directors: Truman Anderson, M.D., Ph. D.
Maurice Mufson, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: (1) To evaluate live attenuated respiratory syncytial virus vaccines in young adults, children and infants. (2) To continue epidemiologic surveillance of lower respiratory tract diseases among infants. (3) To evaluate the small-volume immunoabsorption method for isolation of IgA from selected specimens.

Major Findings: Surveillance of virus, mycoplasma and bacterial infections among infants requiring hospitalization at the Cook County Hospital for acute lower respiratory tract diseases has continued. To date, approximately 2000 infants have been studied. Virus infections, expecially myxovirus infections, were detected in nearly one-half of the ill infants, Mycoplasma pneumoniae infections in about 1.5% and pathogenic bacterial infection in about 5%. Respiratory syncytial virus and parainfluenza 3 virus infections predominated. Outbreaks of respiratory syncytial virus have occurred annually, slightly later each succeeding year; parainfluenza 3 has occurred endemically in most weeks of the study. Pneumococcal bacteremia was detected in about 1% of the infants. Overall, the case fatality rate for respiratory tract diseases was 0.7%.

Vaccine evaluation studies were conducted with a live attenuated respiratory syncytial virus vaccine (grown at 26°C) in adults and children. It proved safe and infective. Pneumococcal polysaccharide vaccine, type II, evaluation was begun in infants and children.

Significance to NIAID Program & Bio-Medical Research: Epidemiological information gathered by the contractor over the years has established the significance of respiratory syncytial virus and the parainfluenza viruses in the etiology of lower respiratory tract disease among infants.

Proposed Course of Project: To terminate on June 30, 1971.

Date Contract Initiated: Contract PH43-63-76 was initiated on November 23, 1962. Contract number was changed to NIH-69-2092 on June 1, 1969.

Current Annual Level: $53,000

HUNTINGDON RESEARCH CENTER, INC. - (NIH-69-2219)

Title: Production of *Mycoplasma pneumoniae* Vaccine

Contractor's Project Director: Theordore R. Carski, M.D.

Project Officer: Robert Purcell, M.D.

Objectives: To produce a 10,000 dose lot of *M. pneumoniae* vaccine and two 1,000 dose lots of *M. pneumoniae* vaccine.

Major Findings: The third lot of inactivated *M. pneumoniae* vaccine prepared under this contract was found to seroconvert 100% of seronegative vaccinees. The *M. pneumoniae* vaccine consisted of a low passaged seed strain of *M. pneumoniae* (Cl-8) grown in HEPES buffered medium. Another 1,000 dose lot of vaccine prepared in the same way and exhibiting similar characteristics is awaiting clinical evaluation. The large 10,000 dose lot of vaccine is being prepared at the present time for use in field trials to determine efficacy of the vaccine.

Significance ot NIAID Program & Bio-Medical Research: An effective *M. pneumoniae* vaccine is needed that is sufficiently antigenic to give high levels of protection against natural disease.

Proposed Course of Project: Contract will continue through December, 1971.

Date Contract Initiated: June, 1969. Previously, it was a supplement to PH43-68-1516 of the Research Resources Branch.

Current Annual Level: $65,000

INDIAN HEALTH SERVICE, HEALTH SERVICES AND MENTAL HEALTH ADMINISTRATION

(Intra-agency Agreement #70 A-11)

Title: Epidemiology of Pneumococcal Infection in the Navajo Indian Reservation

Contractor's Project Director: George E. Bock, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: To conduct clinical and laboratory surveillance among 30,000 Navajo Indians and establish an incidence rate for pneumococcal pneumonia, otitis media and meningitis in preparation for a pneumococcal polysaccharide vaccine field trial.

Major Findings: Pneumococcal infection is an important cause of serious lower respiratory tract illness and otitis media in the Navajo population. Studies were centered at two Public Health Service hospitals in Gallup, New Mexico and Fort Defiance, Arizona. From July 1, 1970 through January 31, 1971, there were twenty-six cases of putative pneumococcal pneumonia at the Gallup Hospital and twenty-two cases at the Fort Defiance Hospital. The rates per thousand population in the Gallup Service Unit for hospitalized pneumonia, extrapolated to a full year were 2.26 for pneumococcal pneumonia. A similar rate for the Fort Defiance Hospital was 2.10 cases per thousand. Otitis media is a major problem among Navajo children. In a retrospective study of acute otitis media during FY 1971, the annual rate per thousand individuals was 143.

Significance to NIAID Program and Bio-Medical Research: This program will define the rate of pneumococcal infection in a population that has a relatively high incidence rate of pneumonia and otitis media. This population has great potential for field trials to demonstrate efficacy of a pneumococcal polyvalent vaccine.

Proposed Course of Project: This intra-agency agreement will be reviewed and evaluated for renewal at the end of the 1971 fiscal year.

Date Intra-agency Agreement Initiated: July 1, 1969.

Current Annual Level: $62,000.

UNIVERSITY OF KENTUCKY - (NIH-70-2265)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: Abram S. Benenson, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: Percutaneous vaccination of 63 children evoked 44 primary reactions. Only 11 had temperatures over 100.4°F for a total of 22 febrile days; 8 had a temperature over 100.9°F for a total of 18 fever-days. Intercurrent infections are responsible for some of these fevers. Three children developed typical erythema multiform with essentially afebrile courses. Two typical reactions were definitely delayed in appearing, reacting their maximal on day 16 and 17. Serological responses have correlated well with the skin reactions, with rises in both hemagglutination and neutralizing antibodies even in the delayed reactors. Six children developed abortive reactions; none of these whose sera have been studied to date have shown a significant antibody response.

Significance to NIAID Programs & Bio-Medical Research: There is currently much concern with the smallpox vaccine due to the infrequent resulting complications. There are some who advocate abolishing compulsory vaccination. This would result in possibly greater problems. A more practical solution would be to get a better vaccination procedure. The Infectious Disease Branch has initiated this study as an immediate step toward this goal.

Proposed Course of Project: Contract has been renewed until December 29, 1972.

Date Contract Initiated: June 30, 1970

Current Annual Level: $50,000

LEE-MAC - (NIH-71-2198)

Title: Production of interferon in a unique cell culture system

Contractor's Project Officer: Thomas McCoy, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: If exogenous interferon proves to be effective in prophylactic and/or therapeutic use in man an economically feasible method of producing interferon is needed. This is a feasibility study to determine whether a unique cytogenerator can be used for this purpose.

Major Findings: Since this is a new contract no data are reported.

Significance to NIAID Programs & Bio-Medical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available exogenous, prepared *in vitro*, and endogenous through use of inducers. There is currently no successful method of economically producing large quantities of human interferon produced in cells other than human leucocytes. This contract is an attempt to determine whether a unique cytogenerator can overcome this obstacle.

Proposed Course of Project: To be reviewed, and results evaluated. If technique proves feasible attempts will be made to scale up, if results are negative contract will be terminated.

Date Contract Initiated: June 1971

Current Annual Level: $50,000

ELI LILLY AND COMPANY (PH-43-68-1280)

Title: Production of Specific Immunogenic Polysaccharide Vaccines from Selected Serological Types of *Streptococcus pneumoniae*

Contractor's Project Director: Robert S. Baker, Ph.D.

Project Officer: Richard E. Horton, M.D.

Objectives: To produce at least 25 gms each of fourteen serotypes of pneumococcal polysaccharide.

Major Findings: During the past year, the contractor has met his quota of bulk produced antigen for types 1, 3, 4, 7, 8, 12 and 14. These materials and individual lots of types 2 and 5 have passed the chemical and biological potency specifications as set forth in the contract. One thousand immunizing doses of types 1, 2, 3, 5, 7 and 8 were packaged in final containers and made available for clinical evaluation in adult volunteers.

Analysis of the epidemiological data on serotypes of pneumococci responsible for bacteremic disease has shown types 9 and 23 to be increasing in frequency. As a result of these observations, production of these two polysaccharide types was added to the workscope when the contract was renewed, October 1, 1970.

Significance to NIAID Program and Bio-Medical Research: The only source of large quantities of pneumococcal vaccines for field trials.

Proposed Course of Project: The contract is in its terminal year. It is anticipated the contractor will complete the assigned work task by December 31, 1971.

Date Contract Initiated: June 28, 1968.

Current Annual Level: $213,000.

UNIVERSITY OF ILLINOIS - (PH-43-65-1031)

Title: Study of Vaccines for Immunization Against Viral Respiratory Infections

Contractor's Project Director: George Gee Jackson, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To conduct studies of vaccines for viral respiratory diseases in four population groups.

Major Findings: Enteric live adenovirus type 4 vaccine when given to Navy recruits four days after arrival reduced ARD hospital admissions about 50% beginning twelve days after immunization. There were no untoward reactions. Mass immunization of 5,000 recruits in the primary phase of training during an ARD epidemic caused a dramatic reduction within four days and a similar reduction in placebo-treated cohorts. The latter had a resurgence of ARD four weeks later. This (ecologic) effect of live adenovirus vaccine was previously underscribed. Outpatient ARD also was decreased, but not pneumonia. In the absence of epidemic ARD, live adenovirus vaccine had little appreciable effect.

Vaccine induced infection was similar at Great Lakes and San Diego, 90% infected and virus shedding on the fifth through twenty-first days. No significant spread occurred among recruits but did between married partners and among siblings in families. Subcutaneous injection of inactivated triple (3,4,7) adenovirus vaccine produced antibody response in twelve of seventeen subjects. One year later, four of fourteen had persistent antibody. After enteric live adenovirus type 4 vaccine, eight of nine antibody-free subjects who had positive serologic response had persistent antibody at sixteen months. Laboratory studies failed to reveal any characteristics by which the vaccine and natural strains of adenovirus type 4 could be distinguished.

Subunit type 7 adenovirus vaccine produced high levels of neutralizing antibody in nine of twenty volunteers; no booster effect was seen after a second dose. Inactivated whole adenovirus type 7 vaccine produced high antibody levels in seven of nine volunteers.

Parainfluenza 2 challenge in volunteers showed protection against infection, virus shedding, and illness due to serum antibody; nasal antibody was a further adjunct. Parainfluenza type 2 did not induce demonstrable delayed type hypersensitivity in guinea pigs.

Repeated challenge with a live influenza A_2 vaccine caused an appreciable increase in antibody against several strains of A_2 virus. The etiologic agent of a sharp epidemic of respiratory illness during the winter was identified as an inhibtor-sensitive, antibody-avid strain of influenza A_2 similar to the Chicago '67 strain.

Regardless of the concentration of the inoculum $1 \times 10^{2.5}$, $1 \times 10^{3.5}$, or 1×10^{5} $TCID_{50}$, rhinovirus type 29 infected similar proportions of antibody-free volunteers. Forty-two percent of volunteers with pre-existing antibody at a titer of 1:4 or greater were protected abainst rhinovirus type 29 challenge. Attenuation of type 29 rhinovirus was achieved by multiple passage in tissue culture.

Significance to NIAID Program and Bio-Medical Research: Provided populations suitable for vaccine studies.

Proposed Course of Project: Contract was terminated December 31, 1970.

Date Contract Initiated: June 26, 1965.

Current Annual Level: Prior year support

UNIVERSITY OF MARYLAND - (PH43-66-63)

Title: Study of Viral Vaccines in Man

Contractor's Project Director: Richard B. Hornick, M.D.

Project Officer: Robert Chanock, M.D.

Objectives: (1) To provide clinical investigators on the intramural NIAID staff with a closed adult population wunder medical surveillance for use in phase 1 evaluation of various vaccines and antiviral compounds. (2) To clinically evaluate various lots of adenovirus vaccines.

Major Findings: Live, oral adenovirus vaccines, types 1, 2, and 5 induced homotypic antibody responses in the majority of susceptible adult vaccinees. Adenoviruses were recovered from the rectal swab samples. No adverse effects were observed.

Significance to NIAID Program and Bio-Medical Research: Provides a closed adult population, close to NIH, for the evaluation of vaccines and antiviral compounds.

Proposed Course of Project: Contract will be in effect until November 30, 1971. Renewal is anticipated.

Date Contract Initiated: November 10, 1965

Current Annual Level: $47,000

THE MEDICAL COLLEGE OF PENNSYLVANIA - (NIH-70-2172)

Title: Production of Interferon in Human Cell Lines

Contractor's Project Director: Kurt Paucker, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Develop a technique for a more efficient, safe and economically feasible production of human interferon. This contractor will study various strains of diploid and heteroploid cells for the production of interferon. He will also investigate various methods of prolonging the yield of interferon from these cells.

Major Findings: Diploid cell strains respond at least as well as established cell lines to induction of interferon under a variety of conditions. Since the criterion of "normalcy" would appear to make diploid cells more acceptable as a source of interferon only these cells will be evaluated further. Ultraviolet irradiated NDV was found to be a better inducer than viable NDV. This was probably due to reduced cell damage. It was also found that serum could be omitted from the cultures resulting in a cleaner final preparation.

Significance to NIAID Programs & Bio-Medical Research: All the interferon inducers currently available are toxic, therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 1, 1970

Current Annual Level: $34,000

MEDICAL COLLEGE OF PENNSYLVANIA - (NIH-70-2126)

Title: Production of Anti-Interferon Sera and Development of Immunoassays

Contractor's Project Director: Kurt Paucker, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Develop techniques for the production of antisera which will be sufficiently specific to inhibit the effect of interferon.

Major Findings: The mouse interferon antigen has been prepared and immunization procedures initiated. Conditions have been defined to remove antibodies formed against remaining contaminating antigens of the partially purified interferon. Similar methods are being used in removing contaminants from the antigen itself. This project has also established more sensitive methods of detecting antibodies against interferon.

Significance to NIAID Programs & Bio-Medical Research: If a specific antisera to interferon is developed it can be used to develop an immunoassay and more important in studies on mechanism and induction of interferon. When an inhibitory reaction is detected the antisera can be used to determine whether the reaction is due to interferon or some other inhibitory effect.

Proposed Course of Project: Contract has been renewed until May 31, 1972.

Date Contract Initiated: June 1, 1970

Current Annual Level: $44,000

UNIVERSITY OF MICHIGAN - (PH43-66-462)

Title: The Epidemiology of Respiratory Disease in a Total Natural Community and Evaluation of Immunizing Agents

Contractor's Project Director: Arnold Monto, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To provide epidemiological data on respiratory diseases in the Tecumseh study area. To conduct vaccine trials in institutions, schools and/or clinics in the area; selection of the location will depend on the composition of the study group required to answer the questions posed by the particular vaccine.

Major Findings: Meningococcal polysaccharide vaccine was administered with varying booster injections to a group of 31 children. There were rises in antibody titer among all children receiving meningococcal Group C vaccines if results of hemagglutination and fluorescent antibody tests are combined. No response was elicited by a booster injection 2 months after the initial dose, but 30% of the vaccinees did have a titer rise when reinoculated 7 months later.

The Group C vaccine was administered at three different dose levels without booster injections to 65 additional children, ages 2-6. The 5 mcg dose was clearly inferior. Less dramatic differences were found between the 25 and 100 mcg doses. No reactions were observed.

In the surveillance of respiratory illness at Tecumseh, Michigan, calendar 1970 began with a brief peak of disease associated with A2/Hong Kong influenza. Parainfluenza virus type 2 was also isolated at this time. The fall peak of rhinovirus-associated illness occurred in late September, parainfluenza virus type 1 was recovered in November and RS virus in December.

Typing of rhinoviruses isolated over 4 years suggests that in Tecumseh a limited number of serotypes may have been involved in a major portion of such infections. It will be essential to see if the same types recur after a period of absence.

Significance to NIAID Program & Bio-Medical Research: Provides a natural open population for a vaccine study.

Proposed Course of Project: To be evaluated and considered for renewal at the end of the contract period, July 3, 1971. Renewal is probable.

Date Contract Initiated: April 4, 1966

Current Annual Level: $102,000

MT. SINAI SCHOOL OF MEDICINE - (NIH-70-2129)

Title: Purification of Human Interferon

Contractor's Project Director: Panayotis G. Katsoyannis, Ph. D.

Project Director: George J. Galasso, Ph. D.

Objectives: Utilizing crude interferon supplied through the NIH this group will undertake the purification of human interferon. The first year was used in developing techniques toward a more purified material than is currently available. This contract will continue purification studies for the dual purpose of applying the product to man and eventually synthesizing the compound.

Major Findings: Several modes to achieve a preliminary enrichment of the material have been explored. These were: precipitation with ammonium sulfate, and adsorption to and elution from Doucil-25, zeolite, and Amberlite IRP-64. Ammonium sulfate precipitation caused too great a loss of biological activity to make it worth while, zeolite proved effective. Material with a protein concentration of 1.5 mg/ml and a specific activity of 24,000 U/mg was prepared from 100 ml batches of crude interferon using these preliminary techniques. Electrofocusing techniques are being undertaken. This group has also successfully perfected a quick assay (24 hours) for the various fractions produced.

Significance to NIAID Programs & Bio-Medical Research: All the interferon inducers currently available are toxic, therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case a more efficient and economically feasible means of producing interferon must be found. This interferon must then be purified to make it safe for man. If the compound could be synthesized it could be made available at a reduced cost.

Proposed Course of Project: This contract will be continued for approximately a five year period.

Date Contract Initiated: June 8, 1970

Current Annual Level: $148,000

NATIONAL CENTER FOR DISEASE CONTROL - (Intraagency Agreement) - 71A17

Title: Smallpox Serologies

Contractor's Project Director: Bernard Lourie, M.D.

Project Officer: George Galasso, Ph. D.

Objectives: Perform vaccinia hemagglutination-inhibition and neutralization tests on 800-1,000 serums.

Major Findings: Contractor is acting as the reference laboratory for the four collaborative smallpox clinical studies. The reference laboratory is responsible for testing 1/4 of the serum samples and correlating results from the other laboratories. No results are available as yet.

Significance to NIAID Programs & Bio-Medical Research: There is currently much concern with the smallpox vaccine due to the infrequent resulting complications. There are some who advocate abolishing compulsory vaccination. This would result in possibly greater problems. A more practical solution would be to get a better vaccination procedure. The Infectious Disease Branch has initiated the collaborative smallpox study of which this contract is a part as an immediate step toward this goal.

Proposed Course of Project: To continue until smallpox collaborative clinical trial has been completed.

Date Contract Initiated: July 1, 1970

Current Annual Level: $15,000

NATIONAL DRUG COMPANY - (PH43-69-2072)

Title: Research and Development of Respiratory Vaccines (Parainfluenza and Adenoviruses)

Contractor's Project Director: Armand N. DeSanctis, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To prepare and safety test zonal purified ten-fold concentrated inactivated parainfluenza type 3 vaccine.

Major Findings: Soluble antigen adenovirus vaccines, types 1, 2, and 5 were prepared under this contract and clinically evaluated under another contract. It was found that the guinea pig ED_{50} correlated very well with antigenicity of the vaccines in adult humans. An ED_{50} of 1:400 in guinea pigs corresponded with 100% seroconversion in humans, while 1:40 corresponded with 75% seroconversion and 1:17 corresponded with 60% seroconversion.

Three lots of monovalent live high passage parainfluenza virus vaccines have been prepared by National Drug Company and clinically evaluated in adult humans. All three lots consisting of types 1, 2, and 3 did not infect adults, and did not cause any adverse reactions in adults. These vaccines will be evaluated in younger age groups to determine whether or not they are infective and antigenic in young children.

Significance to NIAID Program & Bio-Medical Research: This contract provides the experimental vaccines being assessed at various contractor evaluation centers in the program.

Proposed Course of Project: To terminate on October 15, 1971.

Date Contract Initiated: Contract PH43-63-79 was initiated November 29, 1962. The contract number was changed to PH43-69-2072 in May 1969.

Current Annual Level: $25,000

UNIVERSITY OF NEW MEXICO - (NIH-71-2202)

Title: Epidemiology of Pneumococcal Pneumoniae Among the Navajo Indians

Contractor's Project Director: Robert Oseasohn, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: (1) Direct and coordinate studies to determine the incidence of pneumococcal pneumonia and otitis media in the Navajo Indian population receiving medical health care at the Gallup PHS Indian Hospital, New Mexico. (2) Conduct clinical trials with pneumococcal vaccines for the purpose of ascertaining safety, antigenicity and efficacy in selected volunteers.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs & Bio-Medical Research: These studies will establish the incidence of pneumococcal disease in populations with an exceedingly high frequency of pneumonia and otitis media. This target population has great potential for a large field trial to demonstrate efficacy of a pneumococcal polyvalent vaccine to control pneumococcal disease.

Proposed Course of Project: This contract will be reviewed and evaluated before the first aniversary date, May 1972.

Date Contract Initiated: June 1971

Current Annual Level: $90,000

NEW YORK MEDICAL COLLEGE - (PH43-68-1266)

Title: Therapeutic Use of Inducers of Interferon for Viral Infections of Animal and Human Eyes

Contractor's Project Director: Miles A. Galin, M.D.

Project Officer: Samuel Baron, M.D.

Objectives: This is a study to evaluate the value of polyinosinic·polycytidylic acid, an inducer of interferon, in the treatment of viral diseases of the human eye, primarily herpetic keratoconjunctivitis.

Major Findings: This first phase of the project consisted of the evaluation of ocular and systemic toxicity of Poly (In·Cn) in six blind volunteers treated as in-hospital patients with a total dose of 4,900 micrograms by topical administration. There were no ocular toxic reactions or systemic toxicity as demonstrated by a series of blood studies taken prior to, during and after therapy.

Six patients who had been subject to recurrent herpetic keratoconjunctivitis were evaluated for toxicity of In·Cn when they were in a non-infected state. All ocular and laboratory tests done in the blind controls were repeated in these patients. No toxicity was found.

The patients in the double-blind study of In·Cn or IDU (A or B) were divided into three diagnostic groups (I) Acute herpetic keratoconjunctivitis; treated with In·Cn or IDU; (II) Chronic recurrent herpetic keratoconjunctivitis with epithelial lesions; treated with In·Cn or IDU; (III) Chronic recurrent herpetic keratoconjunctivitis without epithelial lesions, treated with In·Cn or artifical tears (Tearisol) (C or D). Patients in Group I responded well. Patients in Group II became worse. Most patients in Group III had no recurrences. The number of patients and length of time treated were not adequate to fully evaluate the efficacy of In·Cn.

Significance to NIAID Programs & Bio-Medical Research: Polyinosinic·polycytidylic acid is an effective inducer of interferon. This topical treatment, if effective, would solve an important medical disease problem which is common and very painful to the patient. The current therapy, although useful, needs improvement.

Proposed Course of Project: This particular contract is being considered as a terminal year unless larger numbers of patients can be obtained.

Date Contract Initiated: June 20, 1968

Current Annual Level: $71,000

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-69-2222)

Title: Longitudinal Evaluation of Vaccines for the Control of Rubella

Contractor's Project Directors: Saul Krugman, M.D. and Louis Z. Cooper, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To conduct: 1) longitudinal studies of vaccine efficacy and safety, 2) studies of unattenuated (natural) rubella and congenital rubella and 3) to develop improved techniques for characterizing the immunology of rubella infection.

Major Findings: A rubella virus vaccine study in postpartum women was completed at two hospitals.

Registrants at two New York City municipal hospital prenatal clinics were screened for rubella susceptibility by the hemagglutination inhibition (HI) antibody test during an approximate one-year period. Susceptibility rates were 10.5% among 2263 women at Harlem Hospital and 26.5% among 1659 women at Bellevue.

Two hundred eighty-eight susceptible women who subsequently delivered were immunized in the immediate postpartum period with a rubella virus vaccine (HPV-77DE$_5$, HPV-77DK$_{12}$, or Cendehill strain). These women were participating in an acceptable program of contraception for at least three months. This consisted most often of a single injection of medroxyprogesterone acetate, 150 mg. The feasibility and acceptance of routinely vaccinating susceptible postpartum women was demonstrated.

During essentially the same period, following licensure of rubella vaccine, fifty-four women were referred because they were pregnant or became pregnant shortly after receiving rubella vaccine. With rare exception, these women were not pre-tested for rubella susceptibility. The majority of these pregnancies were terminated by therapeutic abortion. A few women elected to carry their pregnancies to term, and their infants are being followed for rubella virus excretion and possible congenital defects.

Studies have been conducted to determine the protective efficacy of rubella immune globulin. A dose of 0.3 mg/kg given within several days of rubella virus challenge protected only 50% of children inoculated.

Several challenge rubella viruses have been evaluated, including the Brown strain and the Lederle 25th passage GML strain. Although both strains regularly produced infection and disease in inoculated children, the virus was only rarely communicable to susceptible sentinel controls. Therefore, it would appear that transmissability of rubella virus is lost at an earlier passage level than its disease-producing potential.

Significance to NIAID Program and Bio-Medical Research: This contract will answer a number of questions concerning immunity following natural infection and immunity following vaccination. The widespread utilization of rubella vaccines makes it mandatory to assure the continued protective efficacy and safety of these vaccines.

Proposed Course of Project: Surveillance studies will be continued until June, 1974.

Date Contract Initiated: June, 1969.

Current Annual Level: Prior year support

VANDERBILT UNIVERSITY - (PH-43-68-1038)

Title: Field Study of Live, Attenuated Rubella

Contractor's Project Director: Robert Quinn, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: 1) To determine the antibody response and the frequency of untoward reactions in children receiving rubella vaccine and 2) to determine if vaccine virus is transmitted from recipients to susceptible, non-recipients within the same household.

Major Findings: A field trial of HPV_{80} vaccube was performed in the first and second grades of twenty-two public and parochial schools of Nashville, Tennessee and vicinity. The trial included blind controls and an ancillary family study in which the investigators knew who the control subjects were. Briefly, 1603 subjects were immunized with HPV_{80} at the outset with 41% of these subjects being considered susceptible on the basis of preliminary serologic data. Of the susceptibles followed for serologic response, 98% had successful immunizations. Among those with intermediate levels of antibody, a boost in HAI titer was frequently observed. No significant difference in symptoms was reported in this trial among susceptible vaccinees when compared with placebo recipients and immune vaccinees. In the family study,one placebo recipient had a seroconversion typical of vaccine virus infection.

In addition to the original objectives, the study produced information on the incidence of natural rubella during the study period; existence of partial immunity to rubella as manifested by titer rises in vaccinees with moderate amounts of circulating antibody; and persistence of HAI antibody induced by HPV_{80} vaccine over a twenty-four-month period.

Significance to NIAID Program and Bio-Medical Research: The contractor contributed important data on vaccine safety and immunogenicity. The populations included in the study are geographically and socio-economically different from other studies.

Proposed Course of Project: The study was successfully accomplished and the contract was terminated on September 30, 1970.

Date Contract Initiated: June, 1968.

Current Annual Level: Prior year support

UNIVERSITY OF NORTH CAROLINA - (PH-43-67-48)

Title: Longitudinal Studies of Acute Respiratory Disease in Children

Contractor's Project Director: Floyd W. Denny, Jr., M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To develop populations of young children who may be followed longitudinally for the purposes of observing clinical and epidemiological factors related to acute respiratory disease caused by specific agents; to provide defined populations for the evaluation of methods of prophylaxis, including the use of vaccines, against specific respiratory pathogens.

Major Findings: Under this contract open populations of children of varying socio-economic backgrounds have been followed longitudinally to observe the incidence of acute respiratory disease.

Surveillance of lower respiratory illnesses of children in the pediatric group practice during the past year revealed a sharp epidemic of Mycoplasma pneumoniae infections which peaked in October and November, 1969. Attack rates were very high in school children. A late spring epidemic of RS virus-associated illnesses produced an unusual illness peak in May, 1970. Parainfluenza virus type 3 was responsible for most illnesses occurring in midwinter. The total illness rate was low but the age and sex distribution of illnesses caused by specific agents conformed to previously established patterns.

Studies of acute respiratory disease occurring in a University-sponsored day care center have continued. The two viruses most commonly isolated during the year were parainfluenza virus types 2 and 3. Respiratory syncytial virus was isolated during late spring. The RS virus outbreak was milder both in terms of illness severity and number of isolates than in previous years. Adenoviruses - especially type 2 - were again important causes of illness in young children. Serologic data is now available on these children to determine the incidence of infection with RS virus and parainfluenza virus type 3. The combined isolation and serologic data suggest that most of the children in the program were reinfected yearly with RS virus.

The study of the serologic types of pneumococci associated with respiratory disease was continued during the 1969-70 respiratory year in both the private patient practice and in the day care center. Eighteen months of observations in the private practice showed that over two-thirds of the pneumococci isolated from patients with lower respiratory disease were types 19, 6, 23 and 14. These four types were consistently the most common through all seasons. Longitundinal surveillance of infants in the day care center is continuing to obtain information on patterns of bacterial shedding and reinfection by pneumococcal types previously carried in the nasopharynx.

The booster effect of a second dose of zonal centrifuged, inactivated influenza A2/HK vaccine was studied in a group of junior high school students and young adults, who had received the first dose of vaccine in December 1968 and the second in January or February, 1970. Almost all persons exhibited a booster response and the geometric mean titer was two to threefold higher than the titers following the initial dose.

Significance to NIAID Program and Bio-Medical Research: It is necessary to have a background of continuous respiratory disease surveillance in order to evaluate methods of respiratory disease prophylaxis.

Proposed Course of Project: The contract has been renewed through December 31, 1971.

Date Contract Initiated: November 14, 1966.

Current Annual Level: $86,000.

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-70-2169)

Title: Methods for the Production of Human Interferon

Contractor's Project Director: Jan T. Vilcek, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Develop a technique for a more efficient, safe and economically feasible production of human interferon. A large number of commercially available cells will be investigated for this purpose. Clones will be obtained from the more promising cells and the enhancing effect of antimetabolites studied.

Major Findings: A systematic study was undertaken to determine the optimal conditions for interferon induction with live and UV inactivated NDV and with poly I·poly C in the FS-1 strain of human diploid cells. This has resulted in a basic system for routine production of between 200 and 320 interferon units/2 ml. The contractor then concentrated on methods to increase the yield. By manipulation of the poly I·poly C inducer and antimetabolites they have succeded in increasing the yield 5-fold. This excellent first step is expected to lead to higher yields by varying cells and induction procedures. The goal is an additional 10-fold increase.

Significance to NIAID Programs & Bio-Medical Research: All the interferon inducers currently available are toxic, therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: May 25, 1970

Current Annual Level: $45,000

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-70-2131)

Title: The Evaluation of Topical, Intralesional and Systemic Interferon and Interferon Inducers in the Prevention and Treatment of the Fibroma Virus - Induced Skin Lesion in Rabbits

Contractor's Project Director: Alvin E. Friedman-Kien, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To develop an animal-model system for examining antivirals against virus diseases similar to those in man.

Major Findings: This rabbit fibroma virus model has been found ideal for viral induced cutaneous lesions. This model provides us with a lesion that can be easily observed and used for the systemic, topical and intralesional administration of antiviral agents. They have determined the toxic dosages for rabbits intralesionally and progressed well on prophylactic and therapeutic treatment with poly I·poly C. Intralesional injection of exogenous rabbit serum interferon suppressed the development of the lesion up to 5 or 6 days after infection. Tilorone was found to be ineffective in rabbits. This group has also developed a herpes cutaneous lesion model in the hairless mouse.

Significance to NIAID Programs & Bio-Medical Research: Once these animal-models are firmly established the Program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison and determining the relative merits of the various substances.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: May 25, 1970

Current Annual Level: $48,000

OHIO STATE UNIVERSITY - (PH-43-67-78)

Title: Influence of Cultural Conditions and Concentration Procedures on the Antigenicity of *Mycoplasma pneumoniae* Vaccines

Contractor's Project Director: Norman Somerson, Ph.D.

Project Officer: Robert Purcell, M.D.

Objectives: 1) To redefine the conditions necessary for optimal growth and harvesting of antigenic *M. pneumoniae* vaccine. 2) To develop a satisfactory process suitable for large scale commercial production of this vaccine. 3) To supervise and consult with Huntingdon Research Center and others on request by the Infectious Disease Branch concerning vaccine production.

Major Findings: An experimental *Mycoplasma pneumoniae* vaccine produced at Huntingdon Research Center under the supervision of Dr. Somerson and Dr. Senterfit (Cornell University) produced 100% serologic conversion in human volunteers who were seronegative prior to injection. This vaccine was devised by Dr. Somerson and differed from the previous two lots produced at Huntingdon Research Center (HRC) in that (1) it was prepared from a low passage mycoplasma seed (2) the seed was from a new isolate which had never been grown on medium containing horse serum and (3) considerably more antigenic mass was present in the vaccine.

At the present time, HRC is producing under this contractor's direction, a large production lot of vaccine.

Suspensions of the FH, PI 1428, CI 3, CI 4, and CI 8 strains of *M. pneumoniae* elicited the same TRI antibody levels (four weeks post-inoculation) in guinea pigs. The CI 8 strain is being used as a substitute for strain FH in the vaccine since (1) it was isolated on medium containing 3% PPLO serum fraction, and has never been exposed to horse serum (2) at a low passage level, it appears to grow as a uniform layer of organisms firmly attached to glass, and (3) concentrated suspensions of CI 8 are reactive in immunodiffusion tests.

Because TRI antibody levels were higher in animals receiving organisms grown in medium containing HEPES than when bicarbonate had been used, the HEPES buffer is being used in processes for preparation of M. pneumoniae vaccines.

Significance to NIAID Program and Bio-Medical Research: There is little known about the antigenic composition of mycoplasma preparations obtained from organisms grown under a wide variety of conditions. Such studies are an important prerequisite for production of a potent *Mycoplasma pneumoniae* vaccine. Although *Mycoplasma pneumoniae* can now be grown to high titer and although vaccine materials are capable of inducing antibody, the vaccines produced previously were not sufficiently antigenic to assure high levels of protection against the natural disease. The new improved methodology for vaccine production devised by the contractor would make a *M. pneumoniae* vaccine commercially feasible.

Proposed Course of Project: This contract has been renewed through January 31, 1972.

Date Contract Initiated: December 6, 1966.

Current Annual Level: $54,000.

UNIVERSITY OF PITTSBURGH - (NIH-70-2128)

Title: Development of Better Rabbit Interferon Standards and Assay Systems

Contractor's Project Director: Monto Ho, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Study the stability and potency of the wet frozen ampouled rabbit interferon standard and the possibility of obtaining a lyophilized standard.

Major Findings: A rabbit reference interferon consisting of 20,000 units per ml (ampoule) was prepared for the Research Reagents Branch (catalogue No. G-019-901-028) of NIAID. This material is quite stable for up to 33 weeks at -70°, with a mean titer of 13,300 units per ml. It is also stable up to six months at 4°, and 180 days at room temperature. It shows some inactivation after 21 days at 37°, and its half life is 26 hours at 56°. It may be lyophilized and stored without loss of activity at 4° for at least 3 months. When diluted 1:100, the reference material is still stable at -70°, -20°, and 4°. In comparison to the regular reference, it is less stable at 22°, 37°, 56° and after lyophilization. Results from other laboratories agree that the regular reference is stable. Titers obtained vary greatly; many of them are lower than the assigned unitage. It is concluded that the NIH material is a useful, stable rabbit reference interferon, although its assigned unitage is probably high.

Significance to NIAID Programs & Bio-Medical Research: The NIAID is currently supplying this rabbit interferon as the international standard, therefore, careful control of its stability must be maintained. Further studies on lyophilization are needed to determine whether the material could be dispersed in this form which would be more desirable.

Proposed Course of Project: It is expected that this contract will be terminated at the end of this contract year.

Date Contract Initiated: June 10, 1970

Current Annual Level: $16,000

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA (PH-43-67-681)

Title: Epidemiology of Pneumococcal Disease in Preparation for Field Trials with Pneumococcal Vaccine

Contractor's Project Director: Robert Austrian, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) To continue to determine, on the basis of wide geographic sampling, the pneumococcal serotypes most often responsible for invasive infection in adults and in children; 2) to ascertain the frequency of bacteremic and of nonbacteremic pneumonia and of otitis media caused by specific pneumococcal serotypes in defined populations; 3) to develop further information concerning the immunologic response of man to pneumococcal infection; and 4) to test available monovalent and polyvalent vaccines of pneumococcal capsular polysaccharides in animals and in man for safety and antigenicity in preparation for field trials of such vaccines.

Major Findings: These studies have continued to gather significant data which characterize the prevalence of pneumococcal disease and predominant serotypes of *Streptococcus pneumoniae* responsible for infection. During the past two years of laboratory surveillance, 599 putative pneumococcal infections, sixty accompanied by bacteremia, have been identified among the 254,000 individuals monitored by the contractor.

Phase I studies with monovalent pneumococcal polysaccharide vaccines (types 1, 2, 3, 7 and 8) have been initiated in over 150 adult volunteers. Development of a radioimmunoassay (RIA) technique for the measurement of the antibody response to purified polysaccharide has successfully continued. Currently, the RIH is providing significant data in the evaluation of the antigenicity of candidate monovalent vaccines.

Significance to NIAID Program and Bio-Medical Research: Epidemiologic analysis of clinical and laboratory data will aid in determining high risk groups that will benefit from immuno-prophylaxis with pneumococcal vaccine.

Proposed Course of Project: The contract will be reviewed and evaluated for renewal at the end of the contract year on May 31, 1972.

Date Contract Initiated: February 6, 1967.

Current Annual Level: $321,000.

ST. LOUIS UNIVERSITY (PH43-64-928)

Title: Structure and Replication of Oncogenic and Nononcogenic Adenoviruses and Respiratory Syncytial Virus and the Mechanism of Viral Oncogenesis.

Contractor's Project Director: Maurice Green, Ph. D.

Project Officer: Robert Huebner, M.D.

Objectives: To perform studies of prototype and vaccine adenovirus strains in order to: (1) provide fundamental information to distinguish between oncogenic and nononcogenic adenoviruses, (2) understand the mechanism of oncogenesis by human adenoviruses, (3) understand the structure and immunological reactivity of adenovirus proteins and prepare protein subunits for possible use in adenovirus vaccine, and (4) purify and study respiratory syncytial virus.

Major Findings: Purified preparations of the 31 human adenoviruses were inoculated into newborn hamsters. Two groups of tumor inducing families were found--Group A, Ad 12, 18, and 31, induced tumors in nearly all hamsters within several months. Group B, Ad 3, 7, 14, 16, and 21 (Ad 11 has not as yet induced tumors in animals) induced turmors in a small fraction of inoculated hamsters. Group C, the remaining adenoviruses, have not induced tumors. Highly and weakly oncogenic groups A and B have distinctive properties, base composition and nucleotide sequence, which permit a clear-cut distinction between these groups and the nononcogenic human adenoviruses. Differences between vaccine and prototype adenovirus strains with regard to physical and chemical properties have not been detected.

The presence of functional viral genes transcribing viral mRNA in all rat and hamster cells transformed by human adenoviruses thus far analyzed suggests that molecular hybridization provides a method for testing adenoviruses as etiological agents of human cancer. A sensitive two-step DNA-RNA hybridization-inhibition test was used for this purpose. In step 1, 0.1 ug of adenovirus DNA immobilized on a 6 mm nitrocellulose filter is annealed with 1-2 mg/ml of RNA isolated from human cancer tissues. In step 2, viral DNA is further annealed with highly radioactive virus-specific RNA prepared from adenovirus transformed cells. Adenovirus mRNA sequences, if present in human cancer RNA, will hybridize with viral DNA. That fraction of viral DNA will then be unavailable for hybridization with highly radioactive adenovirus specific RNA in step 2. Over 200 human cancer RNA specimens have been tested by hybridization-inhibition using labeled viral mRNA from groups A, B, and C. All tests were negative and since viral mRNA within each subgroup shares base sequences, genetic material specific for 13 human adenoviruses in groups A, B, and C has been assayed for. About 10,000 viral mRNA molecules are present in adenovirus transformed cells grown in culture and the hybridization-inhibition test is sufficiently sensitive to detect one-tenth this amount, i.e., 1,000 molecules per cell. But it may be argued that human cancer cells have fewer viral mRNA molecules than do adenovirus transformed cells in culture. More sensitive tests may therefore be needed to decisively rule out adenoviruses as causes of human cancer.

The conditions for the plaque assay of RS virus on Hep-2 monolayers were optimized and used to evaluate different cell systems for the production of RS virus. A more sensitive biological assay using fluorescent antibody staining of infected cells is being evaluated. Suspension cultures of KB are an adequate system for the growth of RS virus. As a routine procedure the medium from cells 48 hours after infection is harvested and two liter amounts are treated with 10% polyethylene glycol (average mol wt 6,000-7,5000) and 0.5 M NaCl to precipitate virus. Virus is purified by equilibrium centrifugation on 15 to 60% sucrose gradients. Three visible bands are usually found but infectious virus is present in highest concentration in the bandat about p=1.18. The heavier bands apparently consist of aggregated virus and cell debris. Purified virus was disrupted and fractionated on Sephadex G-100 columns to give two distinct antigenici components when reacted with antisera to RS virus. Preliminary studies by Dr. L. Senterfit indicate these components to be antigenically distinct and possibly identical to antigens A and B as described by Forsyth, Coates, and Chanock.

Significance to NIAID Program & Bio-Medical Research: The necessity of insuring the lack of an oncogenic potential in human vaccine materials was obvious. Chemical and physical analysis of RS virus, and its components yielded basic data about RS virus that will be useful for vaccine production. Since the goals of the contract have been achieved, the contract was terminated.

Proposed Course of Project: Contract terminated April 16, 1971.

Date Contract Initiated: June 17, 1964

Current Annual Level: $22,000 (3 months)

THE ROCKEFELLER UNIVERSITY - (NIH-71-)

Title: Radioimmunoassay for the Quantitative Assay of Human Anti-Meningococcal Antibodies

Contractor's Project Director: Emil Gotschlich, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: (1) Establish a reference serological laboratory to measure group specific antibody to groups A, B and C meningococcal polysaccharides. (2) Develop a standardized radioimmunoassay (RIA) including reference antigens and sera, to measure serum antibody responses of children and infants to varying doses of meningococcal groups A, B and C polysaccharide vaccines, (3) Correlate quantitative data of the antibody response as measured by the RIA with other serological procedures (HA, fluorescent antibody test, bactericidal assay and the opsonic test that are employed to assess similar immune response.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs & Bio-Medical Research: There is a need to develop a highly sensitive and quantitative serological procedure to measure low levels of antibody in infants and children recovering from meningococcal meningitis and those who have been actively immunized with purified meningococcal polysaccharides. Continued development of the RIA procedure has the potential of achieving this goal. The establishment of a reference serological laboratory is essential for a comprehensive assessment of the antigenic and protective capabilities of meningococcal vaccine for children and infants.

Proposed Course of Project: This contract will be reviewed and evaluated before the first aniversary date, May 1972.

Date Contract Initiated: June, 1971

Current Annual Level: $32,000

UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE - (NIH-70-2133)

Title: Antiviral Substances and Animal Systems

Contractor's Project Director: Lowell A. Glasgow, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To develop an animal-model system for examining antivirals against virus diseases similar to those in man. The study will include cytomegalovirus and Herpesvirus hominis in the mouse and the variables will include: 1) timing and dosage necessary for antiviral activity, 2) interferon response, 3) onset, duration and magnitude of viremia, 4) seeding and multiplication of virus in target organs, and 5) immune response.

Major Findings: The therapeutic efficacy of an oral (Tilorone) and a systemic (Poly I:C) interferon inducer was determined in mice infected with Encephalomyocarditis (EMC) virus. The time of onset as well as the duration of the interferon response peak stimulated by these inducing agents was correlated with the level of protection and effect on the pathogenesis of the infection. Poly I:C (100 ug) rapidly induced peak levels of interferon (3-5,000 units/ml) for a short duration (3-6 hours post inoculation). In contrast, Tilorone induced a delayed response with higher levels (8-10,000 units/ml) for a longer period (12-30 hours post inoculation). Infection of mice by the intraperitoneal route with EMC virus is characterized by viremia (24-120 hours), involvement of target organs (24 hours-death), and finally death. Induction of interferon prior to or early during the course of infection resulted in protection of animals. This protective effect was lost with delay of therapy until onset of the viremic phase.

The correlation of treatment with the viremia and the outcome in individual animals indicated that in 100% of surviving animals, induction of interferon could be correlated with inhibition of a detectable viremic phase. Secondly, the viremia could be delayed and partially suppressed without affecting the outcome of the infection. Finally, failure to completely suppress a detectable viremia was inevitably followed by failure of the therapeutic regimen.

Significance to NIAID Programs & Bio-Medical Research: Once these animal-models are firmly established the Program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the Program a direct means of comparison and determining the relative merits of the various substances.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 30, 1970 Contract transferred to University of Utah in September of 1970

Current Annual Level: $33,000

ST. LOUIS UNIVERSITY SCHOOL OF MEDICINE - (NIH-70-2266)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: James D. Cherry, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization

Major Findings: As of March 12, 1971, 319 patients had been immunized in the Smallpox Study. Of this group, 2 patients were removed from the study because of failure to obtain convalescent-phase serum. All other subjects are up-to-date in regard to their reaction photographs and convalescent-phase blood specimens. Hemagglutination-inhibition antibody studies have been performed on 186 serum specimens in Dr. Cherry's laboratory, and 50 of the same specimens have been run at the Center for Disease Control. All the vaccine used (percutaneous) in 1971 were examined for titer following administration, and it appeared that the values were in the expected range.

Significance to NIAID Programs & Bio-Medical Research: There is currently much concern with the smallpox vaccine due to the infrequent resulting complications. There are some who advocate abolishing compulsory vaccination. This would result in possibly greater problems. A more practical solution would be to get a better vaccination procedure. The Infectious Disease Branch has initiated this study as an immediate step toward this goal.

Proposed Course of Project: Contract has been renewed until December 11, 1972.

Date Contract Initiated: June 12, 1970

Current Annual Level: $77,000

STANFORD UNIVERSITY - (NIH-71-2201)

Title: Methods of increasing resistance of polyinosinic.polycytidylic acid to human serum hydrolytic enzymes.

Contractor's Project Director: Thomas Merigan, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study chemical modifications of the chemical inducer polyinosinic·polycytidylic acid in order to stabilize it against human serum hydrolytic enzymes. Ultimate goal is to obtain an effective, less toxic inducer for use in man.

Major Findings: Since this is a new contract no data are reported.

Significance to NIAID Programs & Bio-Medical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available exogenous, prepared *in vitro*, and endogenous through use of inducers. The inducers available are somewhat toxic including the most promising, poly I·poly C. This contract is an attempt to stabilize the compound so that the dosage can be reduced without affecting or increasing its inducing abilities.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of the contract year. Project should take 2-3 years for completion depending on results of initial phase.

Date Contract Initiated: June 1971

Current Annual Level: $59,000

TULANE UNIVERSITY - (NIH-70-2124)

Title: Factors Influencing the Assay of Interferon

Contractor's Project Director: Jules V. Hallum, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: In order to expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay. Toward this end this contract will examine a variety of conditions such as differences due to volume of interferon used, cell concentration in test system, suspension versus monolayer cultures, type of endpoint and relationship of time between infection and harvest of challenge virus in the assay.

Major Findings: The effect of variables, such as volume, cell types and cell concentration, on the sensitivity of interferon assays were studied. Comparison of the relative sensitivity of different types of endpoints indicate that yield reduction is the most sensitive, plaque reduction the next most sensitive and CPE reduction the least sensitive.

Significance to NIAID Programs & Bio-Medical Research: The methods currently in use to assay interferon are tedious, time consuming and are not as reliable as would be desired. It is expected that this contract will yield information which would enable an investigator to do more assays in a shorter period of time with great reliability. This would encourage workers such as chemists to enter into this field as well as assisting the current investigators with their work.

Proposed Course of Project: It is expected that this project may be completed by the end of this contract year.

Date Contract Initiated: June 22, 1970

Current Annual Level: $35,000

TULANE UNIVERSITY - (PH-43-63-75)

Title: Vaccine Studies in Various Population Groups

Contractor's Project Director: William Mogabgab, M.D.

Project Officer: Albert Kapikian, M.D.

Objectives: 1) To maintain respiratory disease surveillance in dependent children at Keesler Air Force Base with respect to incidence, clinical features, and specific etiology. 2) To determine protective effects of respiratory disease vaccines shown to be needed by the results of surveillance. 3) To characterize new agents including variants of known agents. Bacteria, mycoplasma and viruses will be included. 4) To study antigenicity and protective effects of rubella vaccine in children.

Major Findings: Observations for one year were maintained on 642 children and 238 mothers from military dependent families living in an open community on the Mississippi Gulf Coast. Live attenuated rubella vaccine prepared with the HPV-77 strain in duck embryo cell cultures was administered to 197 and withheld from 160 children without hemagglutination-inhibition antibody. Antibody responses occurred in all vaccinees but thirteen required more than one injection. Levels were relatively stable during the study period and even increased in some without evidence of reinfection. Vaccine virus might have been transmitted to three of ninety-eight children without antibody. Rubella was not observed in vaccinated children. It is postulated that reinfection rubella might occur frequently even in the presence of naturally acquired antibody.

Live oral adenovirus vaccine, types 4 and 7 was administered to trainees at Keesler AFB in January 1970. Antigenicity of each type alone and given together was demonstrated. Interference did not occur. Five thousand men received either type 4 alone or type 4 and 7 simultaneously. From January to June 4500 new arrivals received type 4 and 7 vaccines. Rates of respiratory disease were lower in those receiving both types, but evidence of adenovirus infections was too infrequent for evaluation. A very definite reduction in rate of respiratory illnesses in trainees occurred.

Results of antigenic testing of the first of three lots of inactivated *Mycoplasma pneumoniae* vaccine were obtained. Antibody responses were demonstrable by complement-fixation and TRI neutralization. One vaccine lot was quite antigenic and suitable for field trial evaluation.

Significance to NIAID Program and Bio-Medical Research: Provides populations suitable for vaccine studies.

Proposed Course of Project: This contract will be in effect until December 13, 1971.

Date Contract Initiated: December 14, 1962.

Current Annual Level: $50,000.

UNIVERSITY OF UTAH - (NIH-70-2175)

Title: Study of Interferon and Interferon Inducers in the Prevention of Rabies in Animals

Contractor's Project Director: Burton Janis, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To develop an animal-model system for examining antivirals against virus diseases similar to those in man.

Major Findings: The basic model for study has been established and some early studies completed. Exogenous interferon at a dose of 30,000 units was given at the same site and time as the rabies virus. Observations after one month suggest that this dose will not be protective, and the protective effect of larger doses of interferon is being evaluated. The studies on the protective effect of poly I·poly C in rabies infected rabbits indicate protection after one month's observation. Both of these areas need further study and evaluation. Tilorone was not effective as an inducer in rabbits.

Significance to NIAID Programs & Bio-Medical Research: Once these animal-models are firmly established the Program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison and determining the relative merits of the various substances.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 17, 1970

Current Annual Level: $34,000

UNIVERSITY OF UTAH - (NIH-71-2200)

Title: Mechanisms of hyporeactivity to induction of interferon.

Contractor's Project Director: Lowell Glasgow, M.D.
Douglas W. Hill, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: One of the major obstacles in clinical studies with interferon inducers is the hyporeactive period. After initial induction of the antiviral substance a period of 6-7 days is required before more interferon can be induced using the same inducer. The objective of this contract is devising a methodology to overcome this hyporesponsiveness.

Major Findings: Since this is a new contract no data are reported.

Significance to NIAID Programs & Bio-Medical Research: If interferon proves to be effective as an antiviral for prophylactic and/or therapeutic treatment of viral diseases, it will be important to be able to overcome the hyporesponsive period.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 1971

Current Annual Level: $31,000

UNIVERSITY OF VERMONT - (PH-43-69-91)

Title: Immunogenic and Antigenic Studies of Respiratory Syncytial Virus: Evaluation of Rubella, Respiratory Syncytial Virus and Other Vaccines

Contractor's Project Director: Ben Forsyth, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To complete the immunological studies on RS virus; to study the interferon response in selected infectious diseases; to evaluate RS, adenovirus, influenza and other vaccines in selected populations.

Major Findings: Various laboratory studies with RS virus were conducted. Lymphocytes derived from the peripheral lymph nodes of guinea pigs immunized with RS virus developed delayed hypersensitivity to whole RS virus but not to RS virus antigens A and B when measured by the *in vitro* lymphocyte transformation method. Several RS virus strains were adapted to growth at 28°C and 26°C in WI-38. These strains demonstrated differences in cytopathic effect and in their ability to induce interferon production. RS virus antigens were successfully isolated and purified using a new technique, isoelectric focusing. New chromatographic techniques, including the use of Corning CPG-10 were applied to the preparation of RS virus antigens.

Various respiratory vaccines have been clinically evaluated. Two experimental lots of RS virus vaccine were demonstrated to be safe and immunogenic. Shedding of virus following immunization was demonstrated. Meningococcal types C and A polysaccharide vaccines were tested in children and shown to be nonreactive and immunogenic. Preliminary trials with monovalent pneumococcus types 2, 3, 5 and 8 polysaccharide vaccines were begun. Early results indicated that the vaccines were non-reactive and antigenic.

Significance to NIAID Program and Bio-Medical Research: Understanding of the antigens found in the RS virus will aid the development and characterization of RS vaccines being developed by the Branch. Provides important populations for clinical testing of vaccines.

Proposed Course of Project: This contract will continue until December 31, 1971, and renewal is probable.

Date Contract Initiated: Contracts PH-43-67-76 and PH-43-67-77 were initiated in December, 1966, and combined; and the number changed to PH-43-69-91 in January, 1969.

Current Annual Level: $85,000.

UNIVERSITY OF VIRGINIA - (PH-43-63-1128)

Title: Respiratory Infections in Adults

Contractor's Project Director: Jack Gwaltney, Jr., M.D.

Project Officer: Albert Kapikian, M.D.

Objectives: 1) To obtain scientific information which will define further the etiology and epidemiology of acute respiratory infections in adults and provide bases for the prevention or control of these infections. 2) To clinically evaluate respiratory vaccines.

Major Findings: Surveillance of the etiology and epidemiology of acute respiratory infections was continued on 475 industrial workers during the period March 1, 1969 to August 31, 1970. Seven hundred thirty-five (54%) of 1364 respiratory and combined respiratory gastrointestinal illnesses were sampled, and rhinoviruses were isolated from 160 (21.8%). Respiratory and rhinovirus illness rates for the period were within the range predicted from previous observations.

One hundred seventy-nine of 195 (94%) rhinovirus strains isolated in 1963-1966 have been serotyped. Fifteen strains remain untyped after testing against antisera for all of the ninety numbered rhinoviruses. Neutralization testing for all ninety types in a single microtitration test is done with a combinatorial system of thirteen antisera pools. Fifty isolates from 1969-1970 have been tested in this system and thirty-nine (78%) have been identified. Three Charlottesville rhinoviruses (CH 82, CH 47, and SF 365) have been accepted as prototypes following physical and serological characterization.

Serum pairs collected from insurance employees and their families since 1963 were tested for HI antibody to coronavirus strain OC 43 using antigen grown in suckling mouse brain. Fifteen of 594 (2.5%) serum pairs from individuals with colds and one of 577 (0.2%) specimens from asymptomatic persons showed fourfold or greater antibody rises. OC 43 infections were observed in five of seven years and occurred primarily in winter and early spring months. In January and February 1969, this virus accounted for seven of forty-one (17%) sampled illnesses. Each year 5% of the population had serologic evidence of infection with OC 43 or a related virus.

Influenza vaccine trials were completed in three populations: 400 industrial workers, 105 medical students, and 2200 geriatric patients. Injection of A_2/HK vaccine by jet gun alone evoked a fourfold or greater HI or N response in 81% and 79% respectively of those tested. Introduction of vaccine via the nasopharyngeal route alone resulted in antibody responses in 16% of those vaccinated. When combined immunization was used, results paralleled those observed for parenteral vaccine alone. Total influenza rates (febrile and afebrile) were markedly reduced in employees receiving parenteral vaccine alone or in combination with spray but not for employees given spray vaccine alone.

Sixteen volunteers were injected in the inferior turbinate of the nose with formalin inactivated type 14 rhinovirus antigen. Six of seven subjects who received 1.5 ml or more vaccine reacted with fourfold serum antibody responses, but there were no nasal antibody responses. Eleven intranasally vaccinated volunteers were subsequently challenged with a 300 to 1000 $TCID_{50}$/ml concentration of type 14 rhinovirus. Virus shedding rates were no different in vaccinated volunteers from rates in non-vaccinated controls challenged at the same time.

Significance to NIAID Program and Bio-Medical Research: Provides populations suitable for vaccine studies. Provides epidemiological data to establish whether or not there is a need for a particular respiratory vaccine.

Proposed Course of Project: Contract has been renewed until November 30, 1971.

Date Contract Initiated: June 5, 1963.

Current Annual Level: $64,000.

UNIVERSITY OF WASHINGTON - (PH-43-63-562)

Title: Vaccine Evaluation Surveillance Studies

Contractor's Project Director: J. Thomas Grayston, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: 1) Surveillance in a large health cooperative of viral and mycoplasma agents involved in acute respiratory disease. 2) Development and improvement of laboratory methods for detecting agents and antibody from patient specimen materials. 3) Evaluation of vaccines for acute respiratory disease. 4) Rubella vaccine trials in Taiwan.

Major Findings: Studies of pneumonia of various etiology have been conducted in the membership (presently 130,000) of the Group Health Cooperative of Puget Sound since 1963. Studies of croup and bronchiolitis in children under six were added in 1966. During seven years of study, 7,400 cases of pneumonia were recorded. Twenty per cent of all pneumonia was associated with M. pneumoniae infection. M. pneumoniae pneumonia is endemic in Seattle with an average annual incidence of two per thousand. In 1966-67, an epidemic occurred. At the epidemic peak in January 1967, rates were six times higher than in the endemic period. Children five to nine years old consistently had the highest attack rate (fourteen per thousand per year during the epidemic), while the proportion of all pneumonia due to M. pneumoniae was highest among teenagers. Patients with M. pneumoniae had higher rates of headache, fever, sore throat, skin rash and ear complications, but lower rates of coryza and leukocytosis than patients with pneumonia of other etiologies. Duration of illness increased with age. Treatment with tetracyclines and erythromycin reduced the length of illness, but not the antibody response. Follow-up examination revealed no serious sequelae.

A significant rise in antibody titer was demonstrated to one respiratory virus in sera from 118 of 449 children (under fifteen years) with pneumonia and to more than one virus in fifteen.

Studies of lower respiratory tract disease (pneumonia, croup and bronchiolitis) in children under six years of age indicates that parainfluenza, RS, adenoviruses, influenza A and B, and M. pneumoniae can be implicated in all three syndromes. Pneumonia and bronchiolitis were most frequently associated with RS infection, croup most frequently with parainfluenza. During an epidemic of mumps in Seattle, mumps virus infection was also associated with lower respiratory tract disease in young children in the absence of parotitis.

It was estimated that the A_2/Hong Kong vaccine was 76% effective in protecting from illness in the 1968-1969 winter season. The monovalent influenza B vaccine, given in the same dose, was a disappointment in showing an efficacy of less than 20% and in causing an unacceptably high rate of systemic reactions (25%) when given in a dose of 400 CCA. One year later (January 1970), Seattle again experienced an influenza epidemic, this time caused exclusively by A_2/Hong Kong influenza. In the winter of 1970, infection rate for influenza A_2 was 23%. The suburban area, which the previous year had had the lowest rate, experienced a higher rate (31%) than the urban area (16%). Protection, lasting fourteen months after immunization, was evident by 1) significantly less absenteeism from school 2) 58% reduction in influenza-like illness 3) 72-76% reduction in infection rates as estimated by serologic testing, and 4) 80-83% reduction in the rate of serologically confirmed influenza illness.

The unique epidemiologic situation for rubella on Taiwan was utilized for definitive testing of the protective capacity of the new live attenuated rubella vaccines. When the rubella epidemic began in early 1968, over 5,000 children were involved in studies of HPV-77 vaccines grown in three different cell cultures and studies of the Cendehill vaccine. In a large definitive study, the HPV-77 vaccines were shown to be capable of preventing rubella disease (95% efficacy) and this protection was shown to begin within one incubation period of the natural disease. Additional findings were: while the vaccine was highly efficient in preventing disease, subclinical infection with virus shedding in the throat was a common occurrence in the protected children; both HPV-77 and Cendehill vaccines were shed naturally in the throat for up to fifty days, longer than had been previously demonstrated; HPV-77 vaccine could be mixed and given with live measles and live mumps vaccine without interfering with the protective capability of the rubella vaccine.

The children involved in these studies of live attenuated rubella virus vaccine offer a unique opportunity for follow-up studies of decay of antibody following immunization, the persistence of protection from disease and the occurrence of any possible long-term deleterious effect among various vaccine groups as compared to the control group.

Significance to NIAID Program and Bio-Medical Research: Furnishes epidemiological information regarding respiratory agents for which vaccines are being devised and provides populations for vaccine trials.

Proposed Course of Project: This contract was terminated on April 30, 1971.

Date Contract Initiated: April 1, 1963.

Current Annual Level: $70,000.

UNIVERSITY OF WASHINGTON - (NIH-71-2280)

Title: Vaccine Evaluation and Surveillance Studies on Pneumococcal Disease

Contractor's Project Director: J. Thomas Grayston, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: (1) Provide information on the incidence of pneumococcal pneumoniae and otitis media in a large open population located in and around Seattle, Washington. (2) Continue to develop a monkey model to study experimental infection and immune response with selected pneumococci (types 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 14, 18, 19, and 23) as directed by the Project Officer.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: The experience of a large open population with pneumococcal disease will be defined in terms of a incidence rate, and mortality data. This information is vital to structuring a field test to determine the effectiveness of a polyvalent vaccine to protect immunized individuals.

Proposed Course of Project: This contract will be reviewed and evaluated before the next aniversary date - May, 1972.

Date Contract Initiated: May, 1971

Current Annual Level: $150,000

UNIVERSITY OF WASHINGTON - (PH43-68-1321)

Title: Epidemiology of Pneumococcal Disease

Contractor's Project Director: J. Thomas Grayston, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To conduct laboratory surveillance among 120,000 subjects belonging to the Puget Sound Group Health Cooperative, Seattle, Washington and establish an incidence rate for pneumococcal pneumonia.

Major Findings: Subjects in five university affiliated hospitals and 120,000 members of the Puget Sound Group Health Gooperative have been monitered for pneumococcal pneumonia. The attack rate for pneumonia from all causes has averaged 14 cases per 1000 subjects per year. It has been estimated that about 12% of the total cases of pneumonia were associated with a berterial etiology. Typing of pneumococcal isolates has included more than 700 specimens. The distribution of perdominant serotypes from bacteremia pneumonia closely parallels that of other open population groups. The most frequent serotypes recovered from blood and CSF were types 1, 3, 4, 6, 8, 9 and 19. These account for approximately 63% of the pneumococcal illness. Prosecution of the workscope and progress by the contractor has been satisfactory during the contract year.

Significance to NIAID Programs & Bio-Medical Research: This epidemiological study will provide significant information on the role of Streptococcal pneumoniae in acute lower respiratory tract disease of a typical large open population. In addition, it will provide baseline incidence rates for structuring field trials to evaluate efficacy of pneumococcal polyvalent vaccines.

Proposed Course of Project: This contract was terminated on April 30, 1971, although the basic workscope will be continued in a new contract with the same contractor (NIH-71-2280).

Date Contract Initiated: June 28, 1968

Current Annual Level: $80,000

WASHINGTON STATE UNIVERSITY - (NIH-70-2130)

Title: The Influence of Interferon and Interferon Inducers on the Pathogenesis of Feline Panleucopenia, Feline Respiratory Disease Complex, and on Canine Distemper in the Ferret

Contractor's Project Director: Jeffrey C. Duenwald, D.V.M.

Project Officer: George J. Galasso, Ph. D.

Objectives: To develop an animal-model system for examining antivirals against virus diseases similar to those in man.

Major Findings: The models for study were established and it was determined that the cat and the ferret produce interferon. Assay systems for this interferon were established. The toxic dosages of poly I·poly C for these animals was determined. Tilorone was found to be ineffective in these models.

Significance to NIAID Programs & Bio-Medical Research: Once these animal-models are firmly established the Program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison and determining the relative merits of the various substances.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 1, 1970

Current Annual Level: $47,000

MEDICAL COLLEGE OF WISCONSIN - (NIH-69-2070)

Title: Preservation of Mammalian Interferons

Contractor's Project Director: Sidney E. Grossberg, M.D.

Project Officers: Samuel Baron, M.D.
George J. Galasso, Ph. D.

Objectives: To determine the best methods for the preservation of interferons as standard reference reagents. Human and murine interferons will be studied.

Major Findings: High-titered virus-induced mouse interferon, prepared in L cells and partially purified in various ways, has been freeze-dried under different conditions, with and without stabilizing agents. Variables affecting the lability of freeze-dried interferon preparations have been identified. Interferon has been found to behave unlike all other biological materials routinely lyophilized. The mechanism of this difference would be worthy of grant support. It is expected that a supply of both mouse and human interferon will be prepared for lyophilization and submitted to the Research Resources Branch for distribution.

Significance to NIAID Program and Bio-Medical Research: The requirements for the use of standardized reference reagents is becoming increasingly important in interferon research as well as other areas of medical research. It is important that such reagents maintain a defined reference of potency and stability over a long period of time or decline at a predicatable rate. This contract should furnish information not only of importance to interferon reagent stability but also will supply significant data available to all freeze-dried reference reagent standards.

Proposed Course of Project: It is anticipated that this project will be completed with an additional 6 months phase out period.

Date Contract Initiated: May 29, 1969

Current Annual Level: $42,000

MEDICAL COLLEGE OF WISCONSIN - (NIH-70-2125)

Title: Interferon Assay Systems

Contractor's Project Director: Sidney E. Grossberg, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: In order to support and expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay. Toward this end this contract is examining a variety of cell lines and strains to determine the most sensitive. They will also examine nutritive and environmental culture conditions, methods of enhancing the interferon effect, optimal dose of viral challenge and enhancement of detection technique.

Major Findings: Various parameters were examined in an attempt to fulfill the objectives of the study. The most sensitive system for yield reduction assay has been determined to be the human embryonic lung cell line L-132 and VSV. Replicate titrations of reference interferon preparations reveal great variation in titer from experiment to experiment, emphasizing the desirability for appropriately correcting titers in test samples. A promising new interferon assay based on the reduction in yield of viral neuraminidase has been developed which is highly precise, very rapid and economical.

Significance to NIAID Programs & Bio-Medical Research: The methods currently in use to assay interferon are tedious, time consuming and are not as reliable as would be desired. It is expected that this contract will yield information which would enable an investigator to do more assays in a shorter period of time with great reliability. This would encourage workers such as chemists to enter into this field as well as assisting the current investigators with their work.

Proposed Course of Project: Contract has been renewed until May 31, 1972.

Date Contract Initiated: June 1, 1970

Current Annual Level: $34,000

NIH 69 2109

WYETH LABORATORIES, INC. - (NIH-69-2109)

Title: Development of Adenovirus Vaccines

Contractor's Project Director: Ben Rubin, Ph.D.

Project Officer: Robert Chanock, M.D.

Objectives: 1) To prepare live adenovirus vaccines types 1, 2 and 5 in dosage forms suitable for oral administration to infants. 2) Prepare one lot of DNA-free adenovirus type 5 subunit vaccine. 3) Complete the preparation of one lot of adenovirus type 7 subunit vaccine. 4) Complete the safety testing of adenovirus type 3 vaccines.

Major Findings: Small lots of live oral adenovirus type 7 vaccine were produced and tested. Following the reports of clinical efficacy, large production lots were completed. More than 200,000 recruits have received combined live, oral adenovirus types 4 and 7 vaccines. The vaccines, administered simultaneously, were more than 95% protective against specific adenovirus illness and no untoward reactions occurred.

Small lots of live oral and formalin inactivated vaccines have been completed for adenovirus types 1, 2, 5, 14 and 21. Live, oral adenovirus types 1, 2, 5 and 21 vaccines have been tested for antigenicity and safety in semi-closed or closed adult populations. The vaccines were antigenic, and no untoward reactions were observed.

New techniques for the production of immunologically potent adenovirus subunit vaccines have been developed and evaluated. These subunit vaccines were shown to be free of DNA to the limit of detectability with radiotracer technology.

Significance to NIAID Program and Bio-Medical Research: Adenovirus types 4 and 7 are the principal causes of acute respiratory disease in military recruits. These vaccines developed under the contract are safe, potent and effective. Adenoviruses type 1, 2 and 5 are significant causes of respiratory disease in young children.

Proposed Course of Project: Contract has been renewed until April 25, 1972.

Date Contract Initiated: Contract PH-43-63-80 was initiated on January 9, 1963. The contract number was changed to PH-43-68-1316 on May 26, 1968. On April 26, 1969 the contract number was changed to NIH-69-2109.

Current Annual Level: $114,000.

UNIVERSITY OF WISCONSIN - (NIH-70-2132)

Title: Two Swine Viral Disease Systems for the Study of Interferon and Interferon Inducers

Contractor's Project Director: B. D. Easterday, D.V.M. Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To develop an animal-model system for examining antivirals against virus diseases similar to those in man.

Major Findings: Inhibition of viral replication was observed in porcine tissue culture treated with poly I·poly C up to 6 hours post infection with transmissible gastroenteritis, swine influenza and vesicular stomatitis virus. However, interferon was not detected, this may be due to lack of an adequate assay system. Initial in vivo studies using poly I·poly C, tilorone and pyran inducers have not proved effective. These studies are continuing using higher dosages.

Significance to NIAID Programs & Bio-Medical Research: Once these animal-models are firmly established the Program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison and determining the relative merits of the various substances.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 18, 1970

Current Annual Level: $43,000

YALE UNIVERSITY - (NIH-69-2045)

Title: Latent Virus Infection in Cell Cultures to be Used for the Preparation of Vaccines

Contractor's Project Director: G. D. Hsiung, Ph.D.

Project Officer: Hope E. Hopps, DBS

Objectives: To provide information on: 1) Latent virus infection in certain primate and non-primate tissues; 2) the feasibility of certain cell cultures for production of virus vaccines; and 3) the availability of tissues, free of viral agents obtained from domestic or laboratory animals.

Major Findings: A herpes-like virus (HLV) originally isolated from primary rabbit kidney culture has been characterized by Nesburn. Studies of the incidence of this HLV infection in rabbits obtained from different geographical locations were conducted. Primary kidney cultures prepared from each rabbit were maintained for sixty to seventy days and examined regularly for the appearance of cytopathic effect, the appearance of nuclear inclusions, and/or for fluorescent antibody studies. Serum samples from the same rabbits were examined for neutralizing antibody titers against rabbit HLV. Results of these studies have shown that this HLV infection in rabbits is not common. Neutralizing antibody titers were found in rabbits obtained from one of five commercial sources.

A herpes-like virus was commonly isolated from strain 2 guinea pigs, an inbred strain, but rarely from the Hartley strain. The latter strain, however, was found to be susceptible to experimental infection. Studies were designed to elucidate the mode of transmission of this herpes-like virus in Hartley guinea pigs. Findings suggest that the guinea pig herpes-like virus can be transmitted congenitally as well as by contact and by intranasal and oral inoculation.

Significance to NIAID Programs and Bio-Medical Research: A search for latent agents in tissues which are in use or are of potential use in live virus vaccines constitutes an essential part of the Infectious Disease Program.

Proposed Course of Project: The contract will terminate June 30, 1971.

Date Contract Initiated: June 20, 1969.

Current Annual Level: Prior year support

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA - (PH-43-66-465)

Title: Development of a Live Influenza Vaccine

Contractor's Project Director: Florence Lief, Ph.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: 1) To evaluate in humans the live influenza virus vaccines prepared from S+ and S- substrains of a Hong Kong isolate. 2) To conduct further human trials with fresh A2/2946/S+ vaccine and with vaccines containing S+ and S- substrains of A2/Jap 170/62. 3) To continue studies of local and circulating antibodies following infection.

Major Findings: Candidate vaccines have been prepared from cloned S+ and S- substrains derived from a Hong Kong influenza virus isolate and are presently being evaluated in an adult population.

The S- substrain infected 40% of thirteen vaccinees irrespective of pre-existing antibody titer. Infected vaccinees shed virus from one to five days. All except one of the infected persons showed significant increases in neutralizing antibody titer and some of the vaccinees who did not shed virus also showed significant increases in antibody titer. There was no illness. These findings are in keeping with those of the S- substrain of A2/2946 strain. Trials with the S+ substrain are planned. It remains to be seen whether the S+ substrain of A2/Hong Kong will be more infective than the S- substrain as was true with the S+ substrain of A2/2946.

Cold adapted S+ and S- substrain vaccines of the Hong Kong strain have been prepared. These vaccines are identical to the other S+ and S- substrains of A2/HK except that they were grown at 25°C. Clinical trials in adults with these vaccines are planned. Comparisons will be made among the various S+ and S- vaccines.

Significance to NIAID Program and Bio-Medical Research: In addition to the present licensed inactivated influenza vaccines, there is a need for a better immunizing preparation, that is, a live attenuated vaccine.

Proposed Course of Project: To be terminated on June 30, 1971.

Date Contract Initiated: December 1, 1965.

Current Annual Level: Prior year support

YALE UNIVERSITY - (PH-43-68-1037)

Title: Field Trial and Laboratory Investigations of Immune Responses to Rubella and Its Antigenic Components

Contractor's Project Director: Dorothy Horstmann, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: 1) To conduct a clinical study in an open population with a rubella vaccine prepared in DETC (HPV-77 + 5 Duck) and to extend previous reports on immunogenicity, safety and lack of communicability. 2) To administer various rubella vaccines to adult women in order to compare the incidence of arthritis and arthralgia with each vaccine. 3) To study in depth the serological response of purified rubella virus and associate antigens.

Major Findings: Field trials were carried out, using $HPV_{77}DE_5$ and Cendehill vaccines.

A comparative test among susceptible women indicated that the addition of Immune Globulin (human) did not modify the serologic or clinical responses, and $HPV_{77}DE_5$ induced a higher incidence of arthritis/arthralgia with greater severity and duration of symptoms than did Cendehill. The infrequency of such reactions with Cendehill vaccine was also established in a trial involving immunization of women in the few days post-partum.

In a prospective study of an epidemic in military recruits, resistance to reinfection induced by immunization with Cendehill vaccine was shown to be of a low order compared to that following natural infection. The results indicated the probability of a qualitative as well as a quantitative difference in the immune responses of vaccinees and of individuals infected with wild virus.

In another field study, children who had received RA 27/3 (Plotkin) vaccine some two years earlier were challenged with wild virus (Brown strain). The antibody responses to the vaccine were broader than following other vaccines, they persisted in relatively higher titer, and the children exhibited a greater resistance to reinfection than has been observed with either HPV_{77} derivatives or Cendehill vaccine.

Laboratory investigations centered first on the purification and analysis of the physico-chemical properties of the rubella virion. More recently, the structural polypeptides and glycoproteins of the virus have been examined. The results indicate that the virion is composed of five distinct polypeptides, three or four of which are glycoproteins. The precipitinogens theta and iota were shown to be glycoproteins. Other investigations included a detailed study of the variables affecting rubella hemagglutination, and the development of an improved HAI test which gives reliable and reproducible results.

Significance to NIAID Program and Bio-Medical Research: Testing of rubella vaccines in the open community added to the accumulating amount of data proving the safety and efficacy of rubella vaccines. Their improved HAI test has been established as the standard test by the National Communicable Disease Center.

Proposed Course of Project: The contract terminated September 1, 1970.

Date Contract Initiated: June, 1968.

Current Annual Level: Prior year support

YALE UNIVERSITY - (NIH-70-2123)

Title: The Rapid Radiochemical Assay of Interferon: A Conventional and a Matrix Approach

Contractor's Project Director: Richard A. Goldsby, Ph. D.

Project Officer: George J. Galasso, Ph. D.

Objectives: In order to expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay.

Major Findings: The appecability of the radiochemical measurement of the inhibition of viral RNA synthesis by interferon for a rapid assay of interferon has been demonstrated. Sample handling utilizing complementary matrices has been accomplished permitting the handling of large numbers of samples. The work underway and for the ensuing period will be on establishing the precesion of the technique.

Significance to NIAID Programs & Bio-Medical Research: The methods currently in use to assay interferon are tedious, time consuming and are not as reliable as would be desired. It is expected that this contract will yield information which would enable an investigator to do more assays in a shorter period of time with great reliability. This would encourage workers such as chemists to enter into this field as well as assisting the current investigators with their work.

Proposed Course of Project: It is expected that this contract will be completed and terminated at the end of the contract year.

Date Contract Initiated: April 26, 1970

Current Annual Level: $18,000

ELI LILLY AND COMPANY - (PH43-65-563)

Title: Preparation of Respiratory Syncytial and Parainfluenza Virus Vaccines

Contractor's Project Director: Robert Hull, Ph. D.

Project Officer: Robert Chanock, M.D.

Objectives: (1) To prepare and safety test a concentrated inactivated respiratory syncytial virus vaccine for eventual use in clinical evaluation. (2) To conduct research and developmental studies on RS and parainfluenza viruses for the purposes of finding attenuated strains that might be candidates for vaccine preparation.

Major Findings: Several large lots of RS virus vaccine were prepared, concentrated, inactivated, and partially purified through the use of the zonal ultracentrifuge. The vaccine was not sufficiently antigenic to be considered for clinical evaluation.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus and parainfluenza viruses are the most important respiratory pathogens in young infants. Much research and development has been conducted with inactivated vaccines. These vaccines have not been usable for a variety of reasons including lack of stability and lack of immunogenicity via the parenteral route. Since vaccines to these agents are of the utmost importance, the program is now seeking live, attenuated vaccines.

Proposed Course of Project: Contract will terminate on June 30, 1971

Date Contract Initiated: April 1, 1965

Current Annual Level: Prior year support

CHILDREN'S HOSPITAL MEDICAL CENTER, HARVARD MEDICAL SCHOOL - (NIH-71-2196)

Title: Vaccine Evaluation Surveillance Studies with Hemophilus Influenzae Type b.

Contractor's Project Director: David Smith, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: (1) To serve as a reference laboratory for the microbiological identification and serotyping of Hemophilus influenzae isolated from clinical cases of meningitis and otitis media. (2) Develop a radioimmunoassay to measure antibody response to H. influenzae type b infection and immunization with polyribose phosphate (PRP). (3) Conduct vaccine testing in children with PRP to determine safety and dose response data.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs & Bio-Medical Research: Improved methods are needed for microbiological and serological diagnosis of H. influenzae type b infection of children. A highly sensitive procedure such as the RIA is required to measure antibody response to natural disease and condidate vaccines used in immunization studies. An effective vaccines is needed to give high levels of protection against H. influenzae infections.

Proposed Course of Project: To be reviewed and evaluated prior to end of current contract year.

Date Contract Initiated: June 1971

Current Annual Level: $115,000

RESEARCH FOUNDATION OF CHILDREN'S HOSPITAL, WASHINGTON, D.C. - (PH-43-62-473)

Title: Evaluation of Respiratory Virus Vaccines in Infants and Children

Contractor's Project Director: Robert Parrott, M.D.

Project Officer: Robert Chanock, M.D.

Objectives: 1) To study presumably attenuated viruses as potential vaccines. 2) To study factors in the resistance to and pathogenesis of illness resulting from natural infection with respiratory viruses, particularly RS and parainfluenza viruses. 3) To study the "paradoxical" effect following intramuscular injection of inactivated RS vaccine.

Major Findings: Thirty-nine infants and children six months to thirteen years of age received live RS-A2 (26°C) virus by the oropharyngeal and nasopharyngeal route. RS virus was recovered from twenty-two. Seventeen of these twenty-two showed a significant rise in CF and/or plaque reduction serum antibody. Twelve of those from whom virus was recovered and an additional three from whom virus was not recovered showed a threefold or greater rise of RS nasal neutralizing activity. Thus twenty-six of the total showed some evidence of having been infected with the RS strain. The rate of infection was significantly greater in infants and children under two years of age than in older children. Two infants infected with the vaccine strain had some symptoms of respiratory tract illness although the symptoms were relatively minor.

Six children four to six years of age received RS-A2 TS-1 mutant virus by the oropharyngeal and nasopharyngeal route. None was sick. Two shed the virus. Four had a threefold or greater increase in nasal neutralizing activity but none showed a significant increase in serum antibody.

During a recent period of RS virus prevalence there were no unusual RS virus illnesses in individuals who previously had received inactivated RS virus vaccine or other killed or live vaccines.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus and the parainfluenza viruses are the major known causes of severe respiratory illness in young children.

Proposed Course of Project: To continue to assess experimental vaccines.

Date Contract Initiated: June 8, 1962. A new contract number (NIH-70-2091) was given to this contract on December 7, 1971.

Current Annual Level: See new contract NIH-71-2091

RESEARCH FOUNDATION OF CHILDREN'S HOSPITAL, WASHINGTON, D.C. - (NIH-71-2091)

Title: Evaluation of Respiratory Virus Vaccines in Infants and Children

Contractor's Project Director: Robert Parrott, M.D.

Project Officer: Robert Chanock, M.D.

Objectives: 1) To study presumably attenuated viruses as potential vaccines. 2) To study factors in the resistance to and pathogenesis of illness resulting from natural infection with respiratory viruses, particularly RS and parainfluenza viruses. 3) To study the "paradoxical" effect following intramuscular injection of inactivated RS vaccine.

Major Findings: Since this is a new contract no data are reported.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus and the parainfluenza viruses are the major known causes of severe respiratory illness in young children.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at the end of the contract year.

Date Contract Initiated: December 7, 1971. This contract was previously number PH43-62-473.

Current Annual Level: $143,000

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ABBOTT LABORATORIES

NIH-70-2127; production of crude and partially purified human interferon. ID, 99.

NIH-71-2114; the production and testing of rhinovirus research reference reagents. RR, 33.

PH43-67-1355; production of reference reagents to 56 prototype rhinoviruses. RR, 29.

NIH-69-2052; production of reference reagents to adenoviruses. RR, 22.

ATOMIC ENERGY COMMISSION, OAK RIDGE NATIONAL LABORATORY

Interagency Agreement: MAN - Molecular Anatomy Program; MAID-Molecular Anatomy Infectious Disease Laboratory, RR 39.

ALBERT EINSTEIN COLLEGE OF MEDICINE

NIH-69-2071; quality control and reference laboratory for leukocyte antisera. TI, 49.

PH43-68-1295; preparation of soluble purified H-2 isoantigens. TI, 48.

AMERICAN FOUNDATION FOR BIOLOGICAL RESEARCH, BIO-MEDICAL RESEARCH INSTITUTE

PH43-68-619; short-term maintenance of mammalian kidneys by continuous pulsatile perfusion in vitro and development of techniques for freeze-drying and storage of serum and preservation of lymphocytes. TI, 69.

AMERICAN TYPE CULTURE COLLECTION

PH43-68-1008; Catalogue of Arthropod-borne Viruses of the World. RR, 11.

BAYLOR UNIVERSITY

PH43-68-963; Evaluation of experimental respiratory virus prophylactic and therapeutic agents in normal volunteers. ID, 100.

PH43-68-1246; to conduct a research study leading to the preparation of reference reagents to the cytomegaloviruses. RR, 26.

PH43-68-1044; enterovirus antiserum of equine origin. RR, 24.

BIONETICS RESEARCH LABORATORIES, INC.

PH43-68-1327; a primate pre-clinical test center for immune study, primarily with ALS. TI, 66.

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH

PH43-68-694; studies of potential respiratory vaccine strains and isolation methods. ID, 106.

NIH-69-89; certification testing of enterovirus reference reagents. RR, 23.

NIH-71-2112; the production and testing of rhinovirus research reference reagents. RR, 32.

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CENTER FOR DISEASE CONTROL

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Interagency Agreement; smallpox serologies. ID, 129.

CHILDREN'S HOSPITAL RESEARCH FOUNDATION, COLUMBUS, OHIO

NIH-69-2062; rhinovirus reference center. ID, 108.

CHILDREN'S HOSPITAL MEDICAL CENTER, HARVARD MEDICAL SCHOOL

NIH-71-2196; vaccine evaluation surveillance studies with Hemophilus Influenzae Type b. ID, 172.

CLEVELAND CLINIC FOUNDATION

PH43-68-1298; histocompatibility testing of donors and recipients of organ grafts. TI, 51.

COMMUNITY BLOOD COUNCIL OF GREATER NEW YORK

PH43-67-1334; histocompatibility testing of donors and recipients of organ grafts. TI, 52.

CORNELL UNIVERSITY MEDICAL COLLEGE

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DUKE UNIVERSITY

NIH-71-2335; partial support for participation in WHO histocompatibility testing workshop. TI, 74.

ELI LILLY AND COMPANY

PH43-68-1280; production of specific immunogenic polysaccharide vaccines from selected serological types of streptococcus pneumoniae. ID, 121.

PH43-65-563; preparation of respiratory syncytial and parainfluenza virus vaccines. ID, 171.

FLOW LABORATORIES

NIH-71-2288; production of adenovirus reagents. RR, 21.

GEORGETOWN UNIVERSITY

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PH43-67-745; studies of local antibody in respiratory viral immunity. ID, 114.

HARVARD UNIVERSITY

NIH-69-90; extraction, characterization, and testing of HL-A antigens and immunogenetic studies of the HL-A system. TI, 53.

HEKTOEN INSTITUTE FOR MEDICAL RESEARCH OF THE COOK COUNTY HOSPITAL

PH43-67-76; NIH-69-2092; epidemiological studies of viral respiratory infections in children and vaccine evaluation. ID, 116.

HUNTINGDON RESEARCH CENTER

PH43-68-1516; to prepare and test reference reagents to recognized human and animal mycoplasma types. RR, 28.

NIH-69-2219; production of mycoplasma pneumoniae vaccine. ID, 117.

INDIAN HEALTH SERVICE, HEALTH SERVICES AND MENTAL HEALTH ADMINISTRATION

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THE JACKSON LABORATORY

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JOHNS HOPKINS UNIVERSITY

PH43-66-924; factors related to survival of bone marrow allografts in man. TI, 67.

KUAKINI MEDICAL RESEARCH INSTITUTE

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LEDERLE LABORATORIES

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NIH-69-2043; production and testing of myxovirus reference reagents. RR, 36.

LEE-MAC

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MASSACHUSETTS GENERAL HOSPITAL

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MEDICAL COLLEGE OF PENNSYLVANIA

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NIH-70-2172; production of interferon in human cell lines. ID, 125.

MEDICAL COLLEGE OF VIRGINIA

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MEDICAL COLLEGE OF WISCONSIN

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NIH-70-2125; interferon assay systems. ID, 163.

MICROBIOLOGICAL ASSOCIATES INCORPORATED

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MT. SINAI SCHOOL OF MEDICINE

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NATIONAL DRUG COMPANY

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NAVAL MEDICAL RESEARCH INSTITUTE

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NEW YORK MEDICAL COLLEGE

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NEW YORK UNIVERSITY MEDICAL CENTER

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NIH-70-2131; the evaluation of topical, intralesional and systemic interferon and interferon inducers in the prevention and treatment of the fibroma virus-induced skin lesion in rabbits. ID, 139.

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NORTH AMERICAN BIOLOGICALS, INC.

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OHIO STATE UNIVERSITY

PH43-67-78; influence of cultural conditions and concentration procedures on the antigenicity of mycoplasma pneumoniae vaccines. ID, 140.

PETER BENT BRIGHAM HOSPITAL

PH43-68-1408; operation of the kidney transplant registry. TI, 76.

RESEARCH FOUNDATION OF THE STATE UNIVERSITY OF NEW YORK AT BUFFALO

PH43-67-87; characterization of immunological phenomena accompanying homotransplantation. TI. 72.

RESEARCH FOUNDATION OF CHILDREN'S HOSPITAL, WASHINGTON, D.C.

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PH43-62-473; evaluation of respiratory virus vaccines in infants and children. ID, 173.

ROCKEFELLER UNIVERSITY, THE

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SCRIPPS CLINIC AND RESEARCH FOUNDATION

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NIH-69-2090; purified complement components and complement antisera. TI, 63.

SOUTHWEST FOUNDATION FOR RESEARCH AND EDUCATION

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STANFORD UNIVERSITY

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NIH-71-2336; partial support for participation in WHO histocompatibility testing workshop. TI, 77.

NIH-71-2201; methods of increasing resistance of polyinosinic polycytidylic acid to human serum hydrolytic enzymes. ID, 149.

ST. LOUIS UNIVERSITY

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TULANE UNIVERSITY

NIH-70-2124; factors influencing the assay of interferon. ID, 150.

PH43-63-75; vaccine studies in various population groups. ID, 151.

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UNIVERSITY OF AARHUS

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UNIVERSITY OF CALIFORNIA

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NIH-71-2334; partial support for participation in WHO histocompatibility testing workshop. TI, 75.

NIH-70-2183; to evaluate methods for the production of specific dengue immune reagents. RR, 18.

PH43-68-1253; testing and certification of three mosquito blood meal antisera. RR, 17.

NIH-71-2195; stabilization of polyinosinic polycytidylic acid against serum hydrolytic enzymes. ID, 105.

NIH-71-2203; an indirect hemagglutination test to measure antibody response to pneumococcal polysaccharides. ID, 104.

NIH-70-2263; smallpox vaccine evaluation. ID, 103.

NIH-70-2171; production of interferon in cultures of human cells. ID, 102.

UNIVERSITY OF CHICAGO
UNIVERSITY OF IOWA

PH43-69-27/NIH-70-2011; study of the capillary migration assay. TI, 54.

UNIVERSITY OF CHICAGO

PH43-68-83; immunization of humans with group A streptococcal M proteins. ID, 107.

UNIVERSITY OF COLORADO

PH43-62-477; respiratory virus vaccine evaluation and surveillance. ID, 109.

NIH-70-2264; smallpox vaccine evaluation. ID, 110

UNIVERSITY OF FLORIDA

NIH-70-2170; production of human interferon. ID, 113.

UNIVERSITY OF ILLINOIS

PH43-65-1031; study of vaccines for immunization against viral respiratory infections. ID, 122.

UNIVERSITY OF KENTUCKY

NIH-70-2265; smallpox vaccine evaluation. ID, 119.

UNIVERSITY OF LEIDEN

PH43-65-992; search for specific HL-A sera and improvement of histocompatibility techniques. TI, 58.

UNIVERSITY OF MARYLAND

PH43-66-63; study of viral vaccines in man. ID, 124.

UNIVERSITY OF MICHIGAN

PH43-66-462; the epidemiology of respiratory disease in a total natural community and evaluation of immunizing agents. ID, 127.

UNIVERSITY OF MINNESOTA

PH43-68-1303; evaluation of tissue typing methods in organ transplantation. TI, 62.

PH43-68-99; preservation of multicellular organisms and whole organs in vitro with emphasis on the canine at sub-zero temperatures. TI, 71.

UNIVERSITY OF NEW MEXICO

NIH-71-2202; epidemiology of pneumococcal pneumoniae among the Navajo Indians. ID, 131.

UNIVERSITY OF NORTH CAROLINA

PH43-67-48; longitudinal studies of acute respiratory disease in children. ID, 136.

UNIVERSITY OF PENNSYLVANIA, TRUSTEES OF

PH43-66-465; development of a live influenza vaccines. ID, 167.

PH43-67-681; epidemiology of pneumococcal disease in preparation for field trials with pneumococcal vaccine. ID, 143.

UNIVERSITY OF PITTSBURGH

NIH-70-2128; development of better rabbit interferon standards and assay systems. ID, 142.

NIH-70-2184; to evaluate methods for the production of specific dengue immune reagents. RR, 19.

UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE

NIH-70-2133; antiviral substances and animal systems. ID, 147.

UNIVERSITY OF TEXAS

PH43-68-949; suppression of immune responses with drugs and biological products: a search for more effective less myelotoxic immunosuppression. TI, 68.

UNIVERSITY OF UTAH

NIH-71-2200; mechanisms of hyporeactivity to induction of interferon. ID, 153.

NIH-70-2175; study of interferon and interferon inducers in the prevention of rabies in animals. ID, 152.

UNIVERSITY OF VERMONT

PH43-69-91; immunogenic and antigenic studies of respiratory syncytial virus; evaluation of rubella, respiratory syncytial virus and other vaccines. ID, 154.

PH43-67-1188; to test, identify and certify rhinovirus reference reagents. RR, 30.

UNIVERSITY OF VIRGINIA

NIH-71-2113; the production and testing of rhinovirus research reference reagents. RR, 34.

PH43-63-1128; respiratory infections in adults. ID, 155.

UNIVERSITY OF WASHINGTON

NIH-71-2280; vaccine evaluation and surveillance studies on pneumococcal disease. ID, 159

PH43-68-1321; epidemiology of pneumococcal disease. ID, 160

PH43-63-562; vaccine evaluation surveillance studies. ID, 157.

PH43-67-1435; evaluation of tissue typing techniques in clinical homotransplantation. TI, 65.

NIH-71-2280; vaccine evaluation and surveillance studies on pneumococcal disease. ID, 159.

PH43-68-1321; epidemiology of pneumococcal disease. ID, 160

PH43-63-562; vaccine evaluation surveillance studies. ID, 157

PH43-67-1435; evaluation of tissue-typing techniques in clinical homotransplantation. TI, 65.

UNIVERSITY OF WEST INDIES

PH43-67-65; production and testing of arbovirus reference reagents. RR, 20.

UNIVERSITY OF WISCONSIN

NIH-70-2132; two swine viral disease systems for the study of interferon and interferon inducers. ID, 165.

NIH-71-2111; the production and testing of rhinovirus research reference reagents. RR, 31.

VANDERBILT UNIVERSITY

PH43-68-1038; field study of live, attenuated rubella. ID, 135.

WASHINGTON STATE UNIVERSITY

NIH-70-2130; the influence of interferon and interferon inducers on the pathogenesis of feline panleucopenia, feline respiratory disease complex, and on canine distemper in the ferret. ID, 161.

WYETH LABORATORIES, INC

NIH-69-2109; development of adenovirus vaccines. ID, 164.

YALE UNIVERSITY

PH43-68-1037; field trial and laboratory investigations of immune responses to rubella and its antigenic components. ID, 168.

NIH-70-2123; the rapid radiochemical assay of interferon; a conventional and a matrix approach. ID, 170.

NIH-69-2045; latent virus infection in cell cultures to be used for the preparation of vaccines. ID, 166.

YALE ARBOVIRUS RESEARCH UNIT

PH43-66-551; production of arbovirus grouping ascitic fluid. RR, 15

PH43-66-460; testing and identification of arbovirus reagents. RR, 14

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Annual Report of the Assistant Scientific Director for Laboratory and Clinical Research

The administration of the intramural program has remained essentially unchanged during fiscal year 1971 with the Assistant Scientific Director for Laboratory and Clinical Research having primary responsibility for the various laboratories in Bethesda, Chamblee, Georgia; Panama Canal Zone; Hamilton, Montana; and Hawaii. The Scientific Director, Dr. John R. Seal, continues to have the broad responsibility for the administration and direction of both intramural and collaborative research.

For some years the Pacific Research Section of the Laboratory of Parasitic Diseases, located in Honolulu, Hawaii, has been associated with Queens Hospital. The Section is now in the process of moving to newly renovated facilities in Leahi Hospital adjacent to the Department of Tropical Medicine and Medical Microbiology of the University of Hawaii School of Medicine. Funds were recently appropriated to eventually effect the conversion of this School to a full four-year degree-granting medical institution. This new research facility of the intramural program will provide space which has long been needed for the program of the Section. Facilities are now available to accommodate research associates or visiting scientists in this Laboratory with its geographically oriented program involving the disciplines of virology, bacteriology, protozoology, helminthology and entomology. Furthermore, the day-to-day scientific interchange that is now possible with scientists in the Department of Tropical Medicine and Medical Microbiology of the University of Hawaii School of Medicine will be invaluable to our Pacific Research Section. Already the scientific staff of the Section is participating in the teaching activities of the University of Hawaii Schools of Medicine and Public Health.

In early December the Board of Scientific Counselors met in Bethesda to review the program of the Laboratory of Viral Diseases and the Laboratory of Biology of Viruses. It was the unanimous opinion of the Board that the current programs of the two laboratories were up-to-date, vigorous, creative, and of outstanding quality and had great relevance for human health. The emphasis on the molecular aspects of the virus-host interactions provide NIAID with the intramural competence necessary for it to serve as a national resource on human health problems. The Counselors recognized the two laboratories as outstanding examples wherein the NIH has become recognized as a center of excellence and has built a record of success for combining basic and applied research. Dr. Wallace P. Rowe, with his colleagues in the Laboratory of Viral Diseases, was recognized as a leading contributor to research on oncogenic virus. The Board was particularly impressed by the imaginative and capable approaches to the study of slow virus replication and of interactions with the host. They applauded the collaborative slow virus research with the Rocky Mountain Laboratory and hoped that these collaborations would expand. The demonstration of the relationship between oncogenic viruses was considered to be a singular, outstanding achievement. The Counselors felt that the group involved in interferon research had attained a world-wide reputation that, in terms of research on the induction of interferon, was unmatched by any other governmental or academic team. It was pointed out that the NIAID through its efforts in the field of antiviral research can play a unique role in furnishing information on ways to deal with viral infections that are life-threatening. The Board was impressed with the broad biologic views exhibited by Dr. Norman P. Salzman and his staff in the Laboratory of Biology of Viruses. New and exciting techniques are being developed which are opening new vistas in our understanding of the molecular events in virus infection and in cell transformation. Studies in LBV considered to be vital and meritorious included the regulation of viral protein synthesis, the assembly of virus particles, the biochemical characterization of nucleic acids of transformed cells, and the mechanisms of transformation of cells by oncogenic viruses.

The Scientific Counselors recognized the contributions made by the Associates and Staff Fellows to the programs of NIAID This flow of young scientists through the NIH provides challenge and stimulation for the senior professional staff and constitutes an economical means of carrying out high quality and very productive research. There is a continuing demand throughout the country for young scientists trained by people of the caliber of our senior NIAID staff.

In late May the Board of Scientific Counselors meets to review the program of the Laboratory of Infectious Diseases. This will thus complete a review of the major effort of the NIAID intramural program, viral research, within a span of fifteen months. It is becoming increasingly evident as the reviews proceed that NIAID's major intramural component--viral research--is a highly productive effort from which many significant findings related to problems in human health are emerging.

In line with the NIAID's establishment of Allergic Disease Clinical Research Centers in certain areas throughout the country, an effort is being made to establish a group, within the Laboratory of Clinical Investigation, for the study of allergic disorders such as asthma, hay fever, and urticaria. An ad hoc Committee on Allergy, composed of prominent allergists, was formed to advise the Institute concerning the need for such an effort within the intramural program and to recommend established allergists as possible candidates to head up a meaningful program in allergy. The Committee was unanimous that it was highly desirable to set up a program in allergy in LCI as soon as possible.

Dr. Sheldon M. Wolff, NIAID Clinical Director, has made great effort relative to the recruitment of a recognized allergist but to date has been unsuccessful in this attempt. Much of this failure is related to differential between salaries within the NIH and those outside the government for physicians with this specialty. It may be necessary for NIAID's Laboratory and Clinical Research to bring in young physicians who would later develop an imaginative program in allergy. Both the Laboratory of Immunology and the Laboratory of Clinical Investigation have highly competent immunologists who are ready to provide superb and modern support for an allergy program.

With the Institute personnel ceiling being further reduced this year, the most serious problem that has faced Laboratory and Clinical Research has been the inability to replace senior investigators. Certain programs, such as hepatitis, are "ripe" for expansion and only through the addition of adequate personnel can they blossom into significant and highly productive programs. The present personnel reduction policy will have a continuing deleterious effect on important research programs. It is hoped that a mechanism can be found whereby ongoing programs such as hepatitis and new programs such as clinical allergy can be adequately supported by the addition of competent and highly qualified investigators along with technical support personnel. In most cases it has been impossible to reassign professional personnel due to the specialized nature of the areas of research; it usually requires specialists from outside NIAID.

In spite of the lack of adequate professional and technical support in certain areas of the intramural program, the scientists overall have made significant advances in basic and clinical research in subjects relating to allergy and infectious diseases as is reflected in the following reports from the various laboratories of Laboratory and Clinical Research.

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Summary of Program
Laboratory of Clinical Investigation
July 1, 1970 - June 30, 1971

Sheldon M. Wolff, M. D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The Laboratory of Clinical Investigation is about to undergo major organizational changes signifying the final phase of the period of rebuilding begun when the present chief took office. Some of the sections held over from the tenure of the former chief have been abolished. The Clinical Physiology (Head, S. M. Wolff), Medical Virology (Head, J. A. Kasel), and Clinical Allergy and Hypersensitivity (Head, C. H. Kirkpatrick) Sections are to remain. In addition, the Infectious Disease Section has been renamed Clinical Mycology (Head, J. E. Bennett), which more clearly delineates its function. New sections are: Clinical Immunology (Head, M. M. Frank), Inflammatory Diseases (Head, H. R. Kimball), Clinical Parasitology (Head, F. A. Neva) and Biologic Structure (Acting Head, A. S. Rosenthal). The new structure more clearly identifies the present interests and breadth of the staff of the Laboratory.

The continued personnel restrictions have retarded some growth and inhibited the formation of new programs. For example, during the past year an outstanding group of allergists was brought to Bethesda on two occasions under the chairmanship of Dr. John Vaughan. After thorough evaluation and considerable thought, they strongly recommended that a strong program in clinical allergy be initiated in LCI. Following these recommendations, we tried to recruit a senior clinical scientist to head such a program. At least ten men were contacted and a few were offered the position. Unfortunately, some felt that to have a really meaningful program would require more positions and support than could be made available to us under the present circumstances.

As expected, some of our staff that were recruited a few years ago as young investigators are beginning to leave for excellent positions in the academic world. This summer Dr. J. S. Johnson will leave for La Jolla, Dr. R. K. Root for Philadelphia, Dr. J. N. Sheagren for Washington, D. C. and Dr. C. Song for New York City. We are fortunate to have Dr. H. Reynolds returning on July 1 and two of our outstanding younger staff, Drs. D. C. Dale and A. S. Fauci, returning next July 1. Both of the latter men are leaving to serve as chief residents during the coming year.

The members of the Laboratory continue to be productive and receive honors throughout the academic world. Dr. J. Bennett has written a chapter for a new text on infectious diseases. Dr. J. A. Kasel has been invited to take part in a panel at the coming International Virology meeting. Dr. M. M. Frank served as a section chairman at the recent Immunology meetings. Drs. Root, Dale and Wolff have each been invited to present papers at the annual National Foundation Birth Defects Symposium. Dr. Wolff will be the Lilly Visiting Professor in South Africa this coming summer. Space does not permit a number of other significant invitations sent to the members of LCI.

Scientific Developments

The Laboratory continues to place major emphasis on studies relating to clinical immunology, virology, mycology and host defense mechanisms. The role and function of blood cells as major determinants of resistance to infections are extensively investigated.

Neutropenia. Studies have been performed to show that grey collie dogs have a cyclical defect in hematopoiesis and not just in white cells as previously reported. This disease probably represents a disorder of stem cells. Further, it has been clearly demonstrated that patients with malaria have neutropenia, depressed marrow granulocyte reserves and markedly prolonged leukocyte half-lives. On the basis of skin window studies, these investigators have suggested that the number of monocytes a neutropenic patient has may determine his susceptibility to infection. (Dale and Wolff)

Granulomatous Disease. Fifteen patients with Wegener's granulomatosis have been studied. This fatal disease has been successfully treated with cyclophosphamide and the results are now long term. These patients are serving as useful subjects for the study of the effect of cyclophosphamide on granulocyte function and for the investigation of the metabolism of cyclophosphamide. (Fauci and Wolff)

Nonspecific Resistance. The protective properties of bacterial endotoxin against Candida infection in mice have been investigated. Many of the effects of endotoxin can be reversed by iron,and it may be that endotoxin's protective aspects are mediated by its effects on blood iron. (Elin and Wolff)

Inflammation. It has been shown that the cells from Chediak-Higashi mink have a defect on chemotaxis exactly like what they have reported for the cells from human patients with this disorder. (Clark and Kimball)

It has now been possible for granules to be clearly separated from human leukocytes in primary, secondary and probably tertiary granules. (West and Kimball)

White Cell Function. Studies on the functional abnormalities of the white blood cells in the Chediak-Higashi syndrome have been carried out to the point where the defect is clearly defined. These cells ingest bacteria normally but kill gram-positive bacteria at a slower than normal rate, although they kill gram-negative organisms normally. (Root)

Electron microscopic studies have shown that the giant lysosomes do not empty their contents into the phagosomes, which accounts for the killing defect in the Chediak-Higashi syndrome. (Rosenthal)

Cellular Immunity. Investigation has been begun and developed of a unique in vitro model of cellular immunity. Macrophages from peritoneal exudates of guinea pigs in which delayed hypersensitivity to simple protein antigens has been produced are found to be more bactericidal for an unrelated microorganism (Listeria monocytogenes) after overnight incubation with specific antigen. It has been found that the lymphocytes in the exudate from the immune animals can transfer enhanced microbicidal capacity to normal macrophages. This model will, it is hoped, enable the investigators to elucidate how lymphocytes interact with macrophages in this type of immune reaction as well as the metabolic and biochemical changes which must have occurred in the macrophages *per se*. (Sheagren and Simon)

Mycology. Studies have been continued on cellular immunity to cryptococcosis in man. Neutrophils and monocytes from cryptococcosis patients have not exhibited defective intracellular killing of *C. neoformans*. Opsonization has also been found normal. Studies on macrophage-mediated immune responses are now in progress. (Diamond and Bennett)

Quantitative measurement of creatinine assimilation has shown a clear difference between serotype A of *C. neoformans* and serotypes B and C. This is the first biochemical difference *between serotypes* to be demonstrated in this species. Biochemical differences such as this might explain why serotype A is found in different geographic areas than types B and C. Creatinine is of special interest in that it is a major nitrogen source in pigeon droppings, one of the known natural sites for isolation of *C. neoformans*. (Bennett)

Allergy and Hypersensitivity. Two types of defects in lymphocyte function *from patients with chronic* mucocutaneous candidiasis have been identified, and studies of methods of immunologic reconstitution are underway. Two avenues of treatment are under study: viable immunocompetent cells and "transfer factor". The attempts at selective removal of GVH cells for mixed cell populations from experimental animals may ultimately lead to a method of successful and safe reconstitution with allogeneic cells in humans. Other programs are directed at treatment by inhibiting growth of the organism, Candida albicans, but it seems likely that restoration of the integrity of host-defense mechanisms will be necessary to prevent reinfection with this opportunistic organism. (Kirkpatrick and Rich)

Biologic Structure. Visible antigenic markers have been used to characterize *the contribution* of the antibody forming cells and their precursors to antigen mediated lymphocyte proliferation. In contrast to previous views, antibody forming cells and their precursors do proliferate *in vitro*. A new immunoglobulin has been described in the guinea pig, elicited *on foot* pad immunization of HRPO in complete Freund's adjuvant. This immunoglobulin is non-precipitating and inhibitory of rabbit and HRPO precipitin and hemagglutination reaction. The relevance of this new immunoglobulin to *in vitro* models of delayed hypersensitivity is being studied.

Complex lymphoid populations can be shown to consist of two broad classes of cells (though each class probably possesses marked heterogeneity): those involved in antibody production and those participating in cellular immune (delayed hypersensitivity) function. These same investigators have used this segregation of immune effector cells from oil-induced peritoneal exudates. These peritoneal exudate lymphocytes derived from guinea pigs immunized with protein antigens in CFA are highly reactive to two *in vitro* correlates of delayed hypersensitivity, elaboration of macrophage *inhibitory* factor and antigen mediated lymphocyte proliferation. Lymph nodes are less reactive.

This heightened reactivity is independent of the antigen used, not a function of non-lymphoid accessory cells and suggests that the peritoneal exudate lymphocyte is a highly enriched source of cellular immune effector cells. The ultrastructural feature of the lymphocytes is unique and the interaction of antigen with such cells is currently being explored. (Rosenthal and Rosenstreich)

Clinical Immunology. In collaboration with Drs. Ira Green and Leonard Ellman, studies have been performed on the immunologic function in a new strain of guinea pigs with a genetically controlled deficiency of C4. They have been found to have normal delayed hypersensitivity and a defect in antibody synthesis to certain antigens, especially when administered in minimally sensitizing amounts. They have a defect in immune clearance of erythrocytes sensitized with antibody and a defect in bactericidal capacity of their serum. They have normal Arthus reaction and passive cutaneous anaphylaxis reactions. The in vivo studies have been accompanied by a series of in vitro studies, which suggest that these animals are totally deficient in the C4 protein. The in vitro studies demonstrate the existence of an alternate complement pathway which bypasses the early components of complement and allows components to be activated at a point late in the complement sequence. Thus, even in the absence of the early components of complement the inflammatory functions of complement can be manifest. These deficient animals will provide a major new research tool for studies of complement function in health and disease. (Frank)

Previous work by Dr. Frank has suggested that IgM and IgG do not interact with complement by the same mechanisms and that IgG may be capable of producing more damage to a cell surface per complement fixing site than IgM. Recent studies indicate that these differences assume major importance in the in vivo clearance of erythrocytes as seen in autoimmune hemolytic anemia. Their findings indicate that IgM on the surface of an erythrocyte is relatively inefficient in increasing clearance in terms of numbers of complement fixing sites required per cell. When sixty or more IgM C1 fixing sites per cell are on a guinea pig erythrocyte, the cells are cleared from the circulation within five minutes by the liver and then re-released into the circulation where they undergo a normal survival. These cells are not destroyed unless very large amounts of IgM antibody are on their surface. In contrast, IgG coated erythrocytes show decreased survival when as few as 1.4 complement fixing sites are on the cell surface. Such cells are not sequestered by the liver within five minutes but are cleared by the spleen and not re-released back into the circulation. They are phagocytized and destroyed. Both of these effects are complement mediated as shown by parallel studies in the newly described strain of C4 deficient guinea pigs. These studies will place many of the clinical observations noted in patients with autoimmune hemolytic anemia on a molecular basis and may provide an explanation of the function of antibody and complement in these diseases. (Frank and Schreiber)

Medical Virology. Immunization studies have been conducted in man with inactivated influenza virus vaccines, which revealed that current methods of vaccination are relatively inefficient for inducing antibody to the neuraminidase antigen. Among persons never previously exposed to influenza virus there was antibody formation to the enzyme and in individuals with a prior antigenic exposure less than one-half developed a secondary response. Since antibody to the neuraminidase antigen may be important in immunity, these findings indicate that the contemporary approaches to immunization with inactivated vaccines may need revision prior to the appearance of an influenza virus having a new neuraminidase antigen. (Fedson and Kasel)

Parasitology. A series of patients with schistosomiasis, malaria, and a variety of other parasitic diseases have been admitted for evaluation and treatment. Studies on malaria patients are in progress attempting to correlate time of rupture of mature schizonts in the peripheral blood with fluctuations in platelet and white cell counts, complement levels, and onset of fever. In cooperation with the Montgomery County Health Department, a guest worker, Dr. David Hayman, conducted a survey for intestinal helminths and protozoa in a group of recently arrived Cuban immigrants. The results indicated a relatively high prevalence of a number of common intestinal parasites, and plans are in process to study the host-parasite relationship in selected members of this group with Giardia Lamblia infections. (Franck and Neva)

Clinical Pharmacology. Despite the widespread use of pharmacological agents in diseases associated with fever, little is known of the effect of fever itself on the disposal of drugs. The effect of artificial fever induced by administration of etiocholanolone or endotoxin on the metabolism of salicylamide has been studied in volunteers. The major metabolite of salicylamide is its ether glucuronide, and the mechanism of its glucuronidation is believed to be closely related to that by which hepatic glucuronidation of unconjugated bilirubin takes place. It was found that fever consistently caused significant decreases in the rate of glucuronidation of salicylamide. The average proportion of salicylamide excreted as glucuronide decreased from 61% during control to 55% during febrile periods. Patients with Gilbert's disease (studied in collaboration with Drs. Berk and Blaschke) excrete approximately 52% as glucuronide during afebrile periods under identical conditions. Further depression of glucuronidation during spontaneous febrile illnesses in these patients may explain the frequently noted increases in jaundice and unconjugated hyperbilirubinemia. Fever may also significantly delay the metabolism of potentially toxic drugs such as chloramphenicol that are metabolized primarily by means of hepatic glucuronidation. (Song and Wolff)

Canine Immunoglobulins. Further studies with canine IgGd antihapten antibody reactive with the 2,4 dinitrophenyl (DNP) ligand have ruled out univalency and low binding energy as explanations of its failure to precipitate with multivalent antigen. The possibility has been investigated that when binding large antigens this immunoglobulin behaves univalently by examining its interaction with mono-DNP-insulin. The studies have shown that a single molecule of IgGd antibody can bind two molecules of DNP insulin (MW = 6000). However, it is possible that still larger antigens when bound by one antibody combining site will stearically block the other site resulting in functional univalency. If IgGd is functionally univalent, it might be due to "rigidity" of the molecule in the domain of the binding sites. Such rigidity could be due to strong non-covalent interactions or to disulfide bonding between the Fab regions. In the latter case, a disulfide bond linking the Fd portion of the heavy chains or the light chains to one another could impose such a restraint. An inter-light chain bond has been ruled out by studying the distribution of interchain disulfides. IgGd possesses five labile interchain bonds with three between the heavy chains and one between either heavy-light pair.

The primary amino acid sequence of the first 18 residues from the IgGd and IgGa heavy-chain carboxy-termini has been determined, formally establishing these immunoglobulins as IgG subclasses and homologues of IgG of other species. (J. Johnson)

Rheumatoid Factor. The nature of the interaction of human rheumatoid factor (RF) with human and rabbit IgG has been investigated to determine if RF reacts with a native IgG determinant or a non-native determinant(s) exposed by heat aggregatory IgG or by reacting IgG antibody with antigen. The investigators' test system has been the agglutination of Ripley anti D coated r_1, r_1 human erythrocytes with RF. They have tested the ability of native IgG, heat denatured IgG and human and rabbit IgG anti-hapten antibody in the presence and absence of their appropriate univalent haptens to inhibit the agglutination of Ripley cells by RF. Results indicate that native IgG will inhibit agglutination just as effectively as antibody-ligand complexes. Heat aggregated IgG is a more potent inhibitor than native IgG. The data are best explained by conversion of a univalent antigen to a multivalent (aggregated) one and that the RF preferentially reacts with the multivalent antigen due to the favorable energetic advantage of multivalent binding. The importance of this work is in the use of RF as a novel approach to the identification of a possible agent significant to the pathogenesis of rheumatoid arthritis. (Grant and J. Johnson)

Antigenic Competition. Studies of antigenic competition in rabbits have provided evidence of a circulating, nonspecific inhibitor of the immune response. The inhibitory activity is in most instances directly proportional to the strength of the immune response but is not antibody. Similar inhibitory activity has been found in the sera and ascites of mice bearing plasmacytomas. (Fauci and J. Johnson)

Cholera. Studies were initiated to define the mechanisms of resistance to cholera and other bacterial infections of epithelial surfaces. Studies of serum antitoxin in immunized dogs showed neutralizing activity as well as hemagglutinating activity in 19S as well as 7S globulins. This serum provided protection by passive immunization against challenge with live organisms in the dog model of cholera, thus further confirming the importance of humoral antibody in this entirely intestinal disease.

Gastroenteritis. Studies of non-bacterial epidemic gastroenteritis, carried out in collaboration with the LID, demonstrated that this self-limited syndrome was characterized by a brief duration of illness during which xylose and fat absorption, as well as fluid and electrolyte absorption, were abnormal. The transmissible agent appeared to confer immunity in that volunteers were resistant to rechallenge. Further studies are underway to characterize the enzymatic function of the mucosa during the disease and to further define the defect in sugar absorption. (Northrup)

Serial No. NIAID-10(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Office of the Chief

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	3/12			3/12
Professional:	3/12			3/12
Other:	0			0

Project Description:

Objectives:

To develop, direct and coordinate the program of the Laboratory, as defined in the individual research projects; where necessary, to redirect individual projects to meet current needs and advances in the field; to align clinical projects with patient availability; to provide an unexcelled standard of patient care for patients utilized in research.

Methods Employed:

Organize available staff and recruit qualified personnel, both professional and sub-professional, to develop the program and carry out its aims; maintain close liaison with area medical societies, institutions and individuals for referral of patients whose diagnoses fall within the active or proposed disease research categories; the highest standards of patient care are maintained by selection of qualified physicians; the development of necessary policies and continued close and direct supervision of patient care activities; professional consultant services to other institutes in the area of infectious diseases; continued guidance of research projects undertaken by younger investigators; maintenance of staff morale.

Proposed Course:

To continue.

Publications:

1. Lawton, A. R., Asofsky, R., and Mage, R. G.: Synthesis of secretory IgA in the rabbit. II. Production of alpha, light, and T chains by in vitro culture of mammary tissue. J. Immun. 104: 388-396, 1970.

Serial No. NIAID-11(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Clinical Care Program in Support of Research Projects

Previous Serial Number: SAME

Principal Investigators: Dr. Sheldon M. Wolff
Dr. Harvey Simon

Other Investigators: Dr. Edward R. Block
Dr. Joseph S. Bujak
Dr. Lawrence Crane
Dr. Kenneth Dixon
Dr. Fred A. Gill
Dr. DuPont Guerry, IV
Dr. Michael A. Kane
Dr. Joseph E. May
Dr. Theodore E. Nash

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4		11/12	4-11/12
Professional:	4		11/12	4-11/12
Other:	0		0	0

Project Description:

Responsibility for care of patients in the various research areas.

Proposed Course:

To continue.

Publications: NONE

Serial No. NIAID-12(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanisms of Fever and Host Responses

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators:
Dr. Richard K. Root
Dr. Harry R. Kimball
Dr. Anthony S. Fauci
Dr. David C. Dale
Dr. Ronald Elin
Dr. DuPont Guerry, IV
Dr. Alan S. Rosenthal
Dr. John N. Sheagren
Mr. Stanley B. Ward

Cooperating Units:
Dr. David W. Alling, OSD, NIAID, NIH (no project no.)
Dr. Clarence Brown, NCI, NIH (no project no.)
Dr. Paul Carbone, NCI, NIH (no project no.)
Dr. Vincent DeVita, NCI, NIH (no project no.)
Dr. Harvey Gralnick, CC, NIH (no project no.)
Dr. Ralph Johnson, NCI, NIH (no project no.)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4-1/2			4-1/2
Professional:	4-1/2			4-1/2
Other:	0			0

Project Description:

Selected patients are being studied on the wards and in the laboratory. The patients can be divided into the following groups: patients with a) recurrent fever; b) Chediak-Higashi syndrome; c) granulomatous disorders; d) malaria; e) cyclic and other neutropenias; and f) eosinophilia; and normal volunteers.

Extensive studies are in progress relating to the pathogenesis of fever, granulocyte function, immune and other responses of humans and experimental animals to infections, antigens, etc.

Objectives:

Human Studies:

1. To assess varied biologic responses of normal and a variety of patients to etiocholanolone.

2. The effects of pyrogens on the release of vasoactive peptides (kinins) and lysosomal enzymes are under investigation.

3. To assess the responses of humans to multiple doses of endotoxin (endotoxin tolerance).

4. To intensively study groups of patients witn such poorly defined illnesses as familial Mediterranean fever, recurrent fever, Chediak-Higashi syndrome, midline granuloma, Wegener's granulomatosis, cyclic and other neutropenias in an attempt to better delineate the clinical syndrome.

5. To study the mechanisms responsible for the release of endogenous pyrogen.

6. To assess the effect of immunosuppressive agents, in particular cyclophosphamide, on leukocyte function.

7. To study the mechanisms responsible for control of leukocyte levels in health and disease.

8. To define the primary and secondary features of the Cheidiak-Higashi syndrome.

9. To explore the pathogenesis of the increased susceptibility to infection in a variety of diseases.

10. To study what humoral factors, if any, are responsible for the granulocytopenias seen in certain diseases of man.

11. To determine the marrow granulocyte reserves in neutropenic states and to perfect better tests for this parameter.

12. To investigate leukokinetics in patients with depressed and elevated granulocyte counts.

13. To characterize the colony stimulating activity found in the urine of neutropenic patients.

Animal Studies:

1. To define the features of the beige mouse which are similar to the Chediak-Higashi syndrome of man.

2. To determine the lipid pattern of cells from affected mice.
3. To assess the protective effects of endotoxin in mice challenged with various mycotic organisms and, if possible, to characterize the humoral and cellular factor(s) responsible for protection.
4. To further characterize the pyrogenic and hematologic reactivity of immunized rabbits challenged with specific antigen.
5. To examine the role(s) of endogenous and leukocytic pyrogen and soluble antigen-antibody complexes in the production of experimental immune fever.
6. To investigate some of the biologic properties of poly I-poly C.
7. To characterize the humoral factors responsible for endotoxin tolerance.
8. To study what humoral factors, if any, are responsible for the cyclic granulocytopenia seen in grey collie dogs.
9. To better define the grey collie syndrome.
10. To determine the marrow granulocyte reserves in grey collie dogs.
11. To determine the kinetics of circulating granulocytes in dogs with depressed and elevated granulocyte counts.
12. To study the role of colony stimulating activity found in dog urine.

Methods Employed:

Patients and volunteers receive varying doses of endotoxin and etiocholanolone. Rectal temperatures are taken and a variety of responses are determined: antibody levels, immunoglobulins, plasma cortisol, growth hormone, serum iron, clotting factors, etc.

The mobilization of peripheral granulocytes in response to etiocholanolone or endotoxin is studied in patients before, during and after chemotherapy.

Basic studies of immune mechanisms in dysproteinemias, Chediak-Higashi syndrome, etc., include quantitative immunoglobulin determinations using agar diffusion methods, immunoelectrophoresis, paper electrophoresis, complement levels, levels of antibody by the bentonite flocculation technique, ability of patient's lymphocytes to transform under various stimuli such as phytohemagglutinin or SLO antigen, and ability of individuals to develop delayed hypersensitivity through challenge with dinitrochlorobenzene on the skin.

Mice are given endotoxin and subsequently challenged at varying time intervals with such infectious agents as Candida albicans, Plasmodium berghei, etc. Donor mice are exsanguinated; the whole sera or fractions are passively transferred to normal mice, which are then challenged with the infectious agent. Iron is given to mice to study its protective role against infections.

Normal New Zealand NIH rabbits are immunized with nonpyrogenic human serum albumin (HSA) or keyhole limpet hemocyanin using a series of intravenous inoculations. Following an appropriate rest period, antibody levels are measured in the serum using bentonite flocculation and precipitation reactions. Soluble antigen-antibody complexes are formed following precipitation of antibody at equivalence by the addition of sufficient antigen in either normal or immune serum.

An automatic temperature recorder is used to obtain rectal temperatures at regular intervals before and after challenge with antigen, complexes or pyrogen. Hematological responses are evaluated by analysis of ear vein blood samples obtained at various times preceeding and following challenge. Circulating pyrogen is assayed by passively transferring serum from challenged, immunized animals into normal recipients. Buffy coat leukocytes are separated from whole, heparinized rabbit blood following centrifugation and washed in phosphate buffered saline. Counts are made on aliquots of the washed leukocytes, and various concentrations of the agents under study are added to the cell suspensions. After incubation for a specific time interval, leukocytic pyrogen is measured by injection of supernatants into normal and endotoxin-tolerant recipient rabbits, and febrile responses are obtained.

To determine what humoral factors are present in neutropenic states, bone marrow cultures are done in both autologous and isologous plasma. Leukokinetic studies are done using radiochromium-labeled white blood cells.

Bone marrow cells are cultured *in vitro*, and the activity of human and dog urine to stimulate colony formation is measured.

Patient material:

Patients are admitted to the Clinical Center, as are normal volunteers, for these studies. In addition, patients from other Institutes participate in these studies.

Major Findings:

Therapy of the accelerated phase of the Chediak-Higashi syndrome with vincristine and prednisone continues to be encouraging.

Long-term suspension cultures of both fibroblasts and lymphocytes from patients and heterozygotes of the Chediak-Higashi syndrome have been established and their ultrastructure has been described.

Patients with recurrent fever of unknown etiology have been found to represent a diverse array of clinical disorders.

Patients with malaria have (temporarily) neutropenia, diminished marrow granulocyte reserves and markedly prolonged granulocyte survival times in the peripheral blood.

Iron can reverse some of the protective effects of endotoxin when given to mice infected with C. albicans.

The administration of aspirin or dexamethasone with endotoxin to volunteers suppresses the fever and clinical symptoms but does not change the granulocyte or adrenal cortical responses. These agents do interfere with the expected growth hormone response to endotoxin.

Patients on long-term cyclophosphamide therapy do not appear to metabolize this drug differently than normal.

The inflammatory response (Rebuck skin window technique) is depressed only when significant granulocytopenia is present.

Resistance to infection in neutropenic patients may be due to the numbers of monocytes present.

Colony stimulating activity (possibly leukopoietin) cycles in grey collie dogs and patients with cyclic neutropenia.

Prednisone by mouth or intravenous cortisol in addition to etiocholanolone and endotoxin are potent stimuli for the release of granulocytes from the marrow granulocyte reserves.

Androgens do not appear to dramatically elevate granulocyte counts in neutropenic patients.

Grey collie dogs have cyclic neutropenia which recurs every 12 days. During the neutropenic phase, marrow granulocyte reserves are depressed. Serum muramidase level fluctuates cyclically and reaches its lowest point about 24 hours before the peripheral granulocyte count is at its lowest level. The presence of myeloid precursors in the bone marrow also follows a cyclical pattern as does the incorporation of tritiated thymidine into bone marrow cells.

Other cells (lymphocytes, reticulocytes, platelets) besides granulocytes also cycle in grey collie dogs, suggesting that the defect in these animals is due to a regularly recurring defect in stem cells.

Cyclophosphamide has produced striking remissions and clinical improvement in patients with Wegener's granulomatosis.

Radiation therapy has proven to be remarkably effective therapy for midline granuloma.

Significance to Biomedical Research and the Program of the Institute:

The large number and wide variety of studies listed above provide considerable new knowledge dealing with host response mechanisms to a wide variety of clinical and experimental diseases and the pathogenesis of fever.

Proposed Course:

The above outlined studies are to be continued and some areas are to be expanded.

Honors and Awards:

Invited lecture at the University of Virginia School of Medicine, Department of Internal Medicine, Charlottesville, Virginia

Publications:

1. Willerson, J. T., Trelstad, R. L., Pincus, T., Levy, S. B., and Wolff, S. M.: Subcellular localization of S. enteritidis endotoxin in liver and spleen of mice and rats. Infection and Immunity 1: 440-445, 1970.
2. Mickenberg, I. D., Root, R. K., and Wolff, S. M.: Leukocytic function in hypogammaglobulinemia. J. Clin. Invest. 49: 1528-1538, 1970.
3. Fauci, A. S., Benacerraf, B., and Wolff, S. M.: Immunogenic properties of human gamma globulin free of endotoxin contamination. Proc. Soc. Exp. Biol. Med. 136: 264-267, 1971.
4. Reynolds, H. Y., Dale, D. C., Wolff, S. M., and Johnson, J. S.: Serum immunoglobulin levels in grey collies. Proc. Soc. Exp. Biol. Med. 136: 574-577, 1971.
5. Wolff, S. M.: Familial Mediterranean Fever (familial paroxysmal polyserositis). In Harrison, T. R. (ed.): Principles of Internal Medicine. New York, McGraw-Hill Co., 1970, pp. 1075-1078.
6. Dale, D. C. and Wolff, S. M.: Skin window studies of the acute inflammatory responses of neutropenic patients. Blood In press.
7. Dale, D. C., Brown, C. H., Carbone, P., and Wolff, S. M.: Cyclic urinary leukopoietic activity in grey collie dogs. Science In press.

Serial No. NIAID-14(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Medical Virology - Clinical Investigations in Viral Infections and Diseases

Previous Serial Number: SAME

Principal Investigator: Dr. Julius A. Kasel

Other Investigators: Dr. David S. Fedson, LCI, NIAID
Miss Margret Huber, LCI, NIAID

Cooperating Units: Dr. Robert B. Couch, Baylor College of Medicine, Houston, Texas
Dr. Robert B. Chanock, LID, NIAID

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4 10/12			4 10/12
Professional:	4 10/12			4 10/12
Other:	0			0

Project Description:

Objectives:

1. To define and evaluate immunologic factors which contribute to host resistance to viral infections.

2. To investigate the feasibility of employing purified structural viral proteins and recombinant viruses that have been developed in the laboratory as vaccines for the control of disease in man.

3. To conduct clinical studies in individuals following experimental induction of nonbacterial gastroenteritis.

4. To utilize the Chincoteague pony as an animal model to study immunologic response to infectious or inactivated virus and to use viral infections as a means to investigate immunological systems.

Methods Employed:

Human adult volunteers receive conventional or recombinant influenza vaccines by contemporary or other routes of administration. Purified adenovirus hexon antigens in different physical forms are given by two different methods. Immunogenicity is determined by measurement of secretory and humoral antibody formations to defined viral envelope antigens by serologic methods and enzyme inhibition tests. In addition, the immunochemical nature of the response is under study.

Intestinal function, bacterial flora and fluid composition are under study in human adult volunteers following artificial induction of nonbacterial gastroenteritis.

Antibody-negative ponies are immunized with adjuvanated equine influenza virus vaccine by different methods of vaccination. Antibody levels to hemagglutinin and neuraminidase antigens are assayed by serologic and biochemical methods.

Major Findings:

Influenza Virus:

Studies in man indicate that induction of antibody to the neuraminidase antigen appear to be dependent on prior experiences with immunologically identical or related influenza viral enzymes. Immunization of hemagglutinin seronegative and neuraminidase seropositive individuals with inactivated aqueous influenza virus vaccines was followed by rises in nasal and serum antibody titer to both viral envelope antigens. However, antibody rises were more frequent to the hemagglutinin protein. In contrast, infection or vaccination of persons lacking antibody to both antigens resulted in antibody formation only to the hemagglutinin antigen.

Investigations in ponies with no known prior exposure to equine influenza viruses suggest inactivated influenza virus vaccines in adjuvant form given topically, parenterally or by combined methods result in the formation of antibody to both known surface antigens of the influenza virion.

Adenovirus Hexon Vaccine:

The antigenic capabilities of adenovirus types 1, 2 and 5 alum hexon vaccines were compared to those of the same vaccine in the aqueous form. Fluid vaccines were as effective in forming serum neutralizing antibody as were vaccines containing alum. Moreover, aqueous type 5 vaccine given intranasally stimulated humoral antibody formation, whereas the alum form was relatively non-antigenic. Immunization with both forms of adenovirus types 1, 2 or 5 subunit vaccines by different routes of administration was not followed by measurable secretory antibody formation.

Nonbacterial Gastroenteritis:

During the acute phase of experimentally induced acute infectious nonbacterial gastroenteritis, significant abnormalities in d-xylose absorption occurred in clinically ill patients. Malabsorption was also observed in one clinically well individual three days following administration of inoculum. Impaired function returned to normal within one week of the acute illness. Oral gastrointestinal intubation was employed to define changes in intestinal fluid composition and bacterial flora during the course of illness. Available data indicate that the electrolyte composition of jejunal fluid remained unchanged. There was a slight increase in the numbers of a variety of aerobic and anaeorbic micro-organisms during the acute phase of disease; however, there was no indication that altered bacterial populations were related to the occurrence of chemical illness or changes in intestinal absorption.

Significance to Biomedical Research and the Program of the Institute:

It is highly probable that antibody to the neuraminidase antigen of the influenza virus plays a role in immunity to disease. Data from studies in this report indicate that current approaches to immunization in man with inactivated vaccine may need revision before the appearance of an epidemic strain of influenza virus having a new neuraminidase antigen.

Adenovirus vaccines for use in pediatric populations are not available at the present time. With the induction of serum neutralizing antibody in adults by utilization of purified adenoviral subunits, it may now be possible to provide effective immunization against disease in children.

The finding that malabsorption may occur in the absence of changes in intestinal bacteria in persons experiencing acute infectious nonbacterial gastroenteritis may have important implications for studies of intestinal function not only for this disease but also in chronic illnesses characterized by malabsorption.

Proposed Course:

It is planned to continue and expand the program as stated under objectives.

Honors and Awards: Executive Secretary, Influenza Subcommittee of NIAID Infectious Disease Advisory Committee

Publications:

1. Dolin, R., Blacklow, N. R., DuPont, H., Formal, S., Buscho, R. F., Kasel, J. A., Chames, R. P., Hornick, R. and Chanock, R. M.: Transmission of acute non-bacterial gastroenteritis to volunteers by oral administration of stool filtrates. J. Infectious Diseases. 123: 307-312, 1971.

2. Glade, P. R., Kasel, J. A., Haase, A. T., and Chessin, L. N.: Infectious mononucleosis: inteferon production by established suspension culture derived from peripheral blood leukocytes. In Viamonte, M., Koehler, P. R., Witte, M., and Witte, C. (Eds.): Progress in Lymphology II. Stuttgart, German, Georg Thieme Verlag, 1970, pp. 247-249.

3. Couch, R. B., Douglas, R. G., Jr., Rossen, R., and Kasel, J. A.: Rose of secretory antibody in influenza. Proceedings of the Conference on the Secretory Immunologic System, Vero Beach, Florida, 1969.

4. Fedson, D. S., Fulk, R. B., Huber, M. A., Reisberg, M. A. and Kasel, J. A. Antineuraminidase antibody response in serum and nasal secretions following intranasal or subcutaneous inactivated A2/Hong Kong/68 Influenza Virus Vaccine. Journal of Immunology. In press.

5. Couch, R. B., Douglas, R. D., Jr., Fedson, D. S. and Kasel, J. A.: Correlated studies of a recombinant influenza virus vaccine: III. Protection against experimental influenza in man. Journal of Infectious Diseases. In press.

6. Kasel, J. A., Couch, R. B., Douglas, R. D., Jr.: Antigenicity of alum and aqueous adenovirus hexon antigen vaccines in man. Journal of Immunology. In press

7. Rossen, D. S., Kasel, J. A., and Couch, R. B.: The secretory immune system: Its relation to respiratory viral infection. Med. Progr. in Virol. In press.

8. Fedson, D. S. and Brown, P.: Absence of antibody response in man following initial exposure to A2 influenza neuraminidase. Lancet. In press.

Serial No. NIAID-18(c)
1. LCI
2. Infectious Disease Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Systemic Fungal Disease

Previous Serial Number: SAME

Principal Investigator: Dr. John E. Bennett

Other Investigators: Dr. Richard Diamond
Dr. Edward Block
Dr. Richard Root
Dr. Charles Kirkpatrick

Cooperating Units: Miss Anne Jennings, CP, NIH (Project Number 63)
Dr. Robert Young, NCI, NIH (No Project Number)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4-5/12			4-5/12
Professional:	2-11/12			2-11/12
Other:	1-6/12			1-6/12

Project Description:

Objectives:

1. Define defects in host resistance to cryptococcosis.

2. Improve diagnosis and therapy of mycoses.

3. Study physiology of Cryptococcus neoformans.

Methods Employed:

Human neutrophils were separated from peripheral blood by dextran sedimentation. Blood monocytes were obtained by centrifugation in a Hypaque-Ficoll gradient. Monocytes were converted to macrophages by cell culture. Phagocytosis of C. neoformans was measured by microscopic examination and, with macrophages, by uptake of ^{14}C-labeled cryptococci. Intracellular killing was measured by plate counts.

Major Findings:

1. Cellular immunity in cryptococcosis:

After our discovery that human neutrophils could kill C. neoformans, studies were made of 1) factors influencing cellular fungicidal activity, 2) killing by other blood cells and, 3) possible defects in blood cells of cryptococcosis patients. Results were as follows. Fungi with small capsules were killed equally well by neutrophils and monocytes. With larger capsules, monocytes more efficiently phagocytized and killed cryptococci than did neutrophils. Cryptococcal polysaccharide, in concentrations seen in infected patients' sera, did not alter phagocytosis or killing by neutrophils. Neutrophils from one patient with chronic granulomatous disease exhibited defective cryptococcal killing. This suggested that the myeloperoxidase-peroxide system may be one mechanism by which cryptococci are killed intracellularly. However, azide inhibition of myeloperoxidase caused only a temporary delay in killing, implying that alternate systems are available to the neutrophil. Attempts to explore mechanisms of killing with other supposed intracellular enzyme inhibitors, 1 mM hydrocortisone and 6 mM colchicine, were frustrated by the discovery that these agents blocked phagocytosis of cryptococci. Examination of neutrophils from 13 cryptococcosis patients and monocytes from 6 failed to reveal defects in serum opsonins, phagocytosis or cellular killing. Cells from 9 patients without cryptococcosis but with Hodgkin's disease or on corticosteroid therapy also killed normally. Following these results, studies were begun with human macrophages cultured in petri dishes with lymphocytes. Preliminary data indicate that such cultured macrophages do ingest and kill cryptococci. Phagocytosis and killing seems to be enhanced when the cells are cultured in cryptococcal antigen and when the cells originate from a normal control with a positive skin test to cryptococcin. Studies are in progress to establish the specificity of this effect, as well as to verify preliminary data suggesting impaired killing by cells of cryptococcosis patients.

2. Serology of aspergillosis:

Enthusiastic reports about the diagnostic value of several serologic tests in bronchopulmonary (non-invasive) aspergillosis prompted an investigation of these tests in invasive aspergillosis. This infection presents a difficult and frequent diagnostic problem at N.I.H. and other oncology centers. Unfortunately, neither agar gel precipitin, complement fixation or indirect immunofluorescent tests detected an antibody response in these infected but immunosuppressed patients. The same tests easily demonstrated antibody in bronchopulmonary aspergillosis. This antibody was shown to be γG by Sephadex column chromatography and immunoelectrophoresis. The study was done in collaboration with Dr. Robert Young of NCI.

3. Identification and significance of red yeast-like fungi in clinical specimens:

In collaboration with Miss Anne Jennings in Clinical Pathology, all red yeast-like fungi isolated from clinical specimens during a 29-month period were identified. Of the 45 isolates obtained, 44 represented 5 different species of Rhodotorula and one isolate was Sporobolomyces salmonicolor. The single most common site yielding these isolates was stool, 2.5% of such specimens being found to contain Rhodotorula. No isolate was thought to represent an infection, including 2 from blood, 1 from CSF, 1 from liver biopsy and 1 from a wound. Frozen orange juice supplied to patients was suggested as a source of the most common species, Rh. rubra.

4. Chemotherapy of mycoses in man:

Clotrimazole, a new synthetic drug, was administered to 2 patients, both of whom had failed to respond to other agents. One patient, with chronic mucocutaneous candidiasis, was studied in collaboration with Dr. Charles Kirkpatrick. This patient has shown a marked and prolonged improvement in his infection. Both patients have shown a gradual reduction of blood levels during continued therapy with a given dose, suggesting progressively increased drug catabolism. The candidiasis patient, who is also hypoparathyroid, has shown increased effect from dihydrotachysterol therapy. The possibility is being investigated that this effect, as well as increased drug catabolism, represents increased hepatic hydroxylase activity.

A total of 18 cryptococcosis patients have received a full course of 5-fluorocytosine. Results parallel those reported last year and still indicate that this is a useful drug.

To date, 19 patients with systemic mycosis have been assigned randomly to receive either daily or every-other-day intravenous amphotericin B in a fixed dose regimen. Currently, both regimens appear comparable in toxicity and efficacy.

5. Biologic differences between serotype of C. neoformans:

Quantitative assimilation of creatinine was measured as the growth ratio of the fungus in creatinine as compared to ammonium as the sole nitrogen source. Ratios of serotype B and C isolates were similar and much higher than those of serotype A. Type D isolates fell into 2 distinct groups; those in the range of type A and those with even higher ratios than most B and C isolates. Preliminary data suggest that these subgroups of type D also differ antigenically.

6. Immune complexes in cryptococcosis:

Studies have continued on a rabbit model which appear to parallel the clinical situation in which both cryptococcal antigen and antibody are present together in the blood. When such rabbit sera were handled in a way that did not favor aggregation of γG, neither γG globulin nor anticryptococcal antibody could be found in high molecular weight effluents from Sephadex G200 column chromatography. This supported the conclusion that antigenic heterogeneity rather than immune complexes explained the serologic findings in the rabbit.

Significance to Biomedical Research and the Program of the Institute:

1) Studies on neutrophil and monocyte function have provided important insights into one aspect of host defense mechanisms in cryptococcosis. If current studies on cultured macrophages define a cellular defect in cryptococcosis patients, it might prove possible to elucidate this defect at a molecular level. Not only might such an understanding have therapeutic implications but it might lead to a better definition of who is at risk of acquiring cryptococcosis. Even if no specific defect can be found, the research should improve our knowledge about macrophage-mediated immune responses.

2) The study of red yeast-like fungi in clinical specimens indicated that these micro-organisms were not pathogens in the patients studied.

3) The unique facilities of the Clinical Center continued to permit the acquisition of much-needed information about systemic antifungal agents.

4) The metabolic differences between serotypes of C. neoformans may lead to a better understanding why these serotypes have a different geographic distribution.

Proposed Course:

Studies on cellular immunity in cryptococcosis will be continued and expanded to the experimental animal. The emphasis of chemotherapeutic studies will be to acquire needed information about the pharmacology of 5-fluorocytosine, given either alone or together with amphotericin B. The studies on Aspergillus serology and red fungi have been terminated.

Honors and Awards:

Lecturer - Department of Microbiology, Johns Hopkins University School of Medicine.

Member - Advisory Committee on Anti-Infective Agents, Food and Drug Administration.

Publications:

1. Young, R. C., Bennett, J. E., Vogel, C. L., Carbone, P. P., and DeVita, V. T.: Aspergillosis. The spectrum of the disease in 98 patients. Medicine 49: 147-173, 1970.

2. Bennett, J. E.: Clinical applications of serologic tests for systemic mycoses. Modern Treatment 7: 577-580, 1970.

3. Bennett, J. E., and Bailey, J. W.: Control for rheumatoid factor in the latex test for cryptococcosis. Amer. J. Clin. Pathol. In Press.

Serial No. NIAID-23(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Observations on the Canine Immunoglobulins

Previous Serial Number: SAME

Principal Investigators: Dr. John S. Johnson

Other Investigators: Dr. J. Andrew Grant*
Miss Lois Renfer

Cooperating Units: Dr. Peter Z. Allen, University of Rochester,
Dept. Microbiology, Rochester, New York

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	5/12	3/12		8/12
Professional:	5/12	3/12*		8/12
Other:	0	0		0

Project Description:

Objectives:

Construction of a molecular model for canine serum and colostral IgA and for canine IgGd. Elucidation of the mechanism responsible for the lack of precipitation of IgGd antibody with multivalent antigens.

Methods Employed:

Gel filtration and ion exchange chromatography; standard techniques of zone electrophoresis, immunodiffusion; sedimentation velocity and equilibrium ultracentrifugation; equilibrium dialysis and fluorescence quenching for determination of binding energies; liquid scintillation spectroscopy; determination of disulfide bonds by the ^{14}C iodoacetamide method.

Major Findings:

The molecular weight discrepancy between serum and secretory IgA has been substantiated by sedimentation equilibrium centrifugation. The α chain molecular weights are approximately 58,000 and L-chains 23,500.

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* Salaried by Immunology Branch, NCI, NIH.

Secretory IgA contains a third polypeptide component of approximately 50,000 daltons accounting for its additional mass. The molecular models constructed from these data indicate both molecules are composed of covalently linked dimers consisting of 2α and 2 L chains linked by disulfide bonds.

The molecular weight of IgGd has been critically examined by sed. equilibrium and is 148,000 daltons. It contains 2 γ chains of 50,000 daltons MW and 2 L chains of 22,500 daltons MW. Reexamination of disulfide bonds reveals a total of 17 with 5 bonds representing interchain linkages. 3 are inter H and 2 are inter H-L. The L chains are not covalently linked to one another.

Because IgGd antibody will not precipitate with large multivalent antigens,we have suggested this is due to stearic hindrance of one of its binding sites. We have reexamined the binding of DNP-Lys and mono-DNP insulin by anti-IgGd DNP antibody utilizing equilibrium dialysis. The previous conclusions of high binding energy and divalency have been corroborated. That the antibody is capable of binding 2 moles of DNP insulin indicates its interaction with molecules of approximately 6000 daltons is not hindered. However, larger ligands would constitute a more rigorous test. Unfortunately,we have been unable to prepare usable mono DNP antigens in the 60,000 - 70,000 daltons range. Our molecular weight data indicate a basic structure similar to that of precipitating IgG's. However, if one of the inter H disulfides were near the H chain amino terminus in Fd,it would impose a marked restraint on the molecule in the domain of the binding sites. Strong non-covalent interactions could result in the same restraint. We are involved in studies to determine if these restraints exist.

Dr. Grant has determined the C terminal octadecapeptide amino acid sequence of IgGd and IgGa,b and formally proven these are IgG subclasses homologous with IgG of other species.

Collaborative studies with Dr. P. Z. Allen have indicated extensive antigenic relatedness of equine and canine immunoglobulins. Preliminary evidence indicates IgGd and equine IgG(T) may be closely related.

Significance to Biomedical Research and the Program of the Institute:

The studies defining the molecular models of the canine IgA's have made critical testing possible of the role of secretory piece in stabilizing secretory IgA from proteolysis and in serving a transport function. Canine IgGd like equine IgG(T) does not precipitate multivalent antigens and does not fix complement. The elucidation of mechanisms underlying these properties is of important practical interest to those investigators involved in clinical use of antilymphocyte globulin.

Finally, as we have pointed out before, dogs suffer from diseases mimicking closely syndromes found in man. Many of these exhibit prominent immunological derangements. Knowledge of the canine immunoglobulins may be of considerable aid in studying these diseases as models of human disease.

Proposed Course:

The canine IgA work has been submitted for publication. Dr. Allen and I have completed a manuscript on equine-canine homologies and will shortly submit it for publication. Dr. Grant and I have submitted a manuscript on the sequence work. We are finishing the IgGd structure work within the next few weeks and will submit a manuscript for publication.

Except for studies on susceptibility of IgA to proteolysis and examination of these molecules for J chain (recently reported in humans and rabbits) by Dr. Reynolds on his return in August this program will be terminated due to departure of the principal investigator.

Honors and Awards: NONE

Publications:

1. Reynolds, H. Y., and Johnson, J. S.: Quantitation of Canine Immunoglobulins. J. Immun. 105: 698-703, 1970.

2. Reynolds, H. Y., and Johnson, J.S.: Structural Units of Normal Canine Serum and Secretory IgA. Biochemistry. In press.

Serial No. NIAID-25(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Juvenile Rheumatoid Arthritis

Previous Serial Number: SAME

Principal Investigators: Dr. John S. Johnson

Other Investigators: Dr. J. Andrew Grant*

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	5/12	9/12		1-2/12
Professional:	5/12	9/12*		1-2/12
Other:	0	0		0

Project Description:

Objectives:

Studies of the significance of rheumatoid factor and its use as an approach to etiologic factors in arthritis.

Methods Employed:

Gel filtration and ion exchange chromatography; standard techniques of immunodiffusion and zone electrophoresis; analytical and preparative ultracentrifugation; bacterial agglutinations; passive hemagglutination and HI; affinity chromatography.

Major Findings:

Little additional information has been accumulated on patients with JRA due to lack of referrals. We have studied one patient further who exhibited aspirin-induced hepatitis. Upon rechallenge with ASA he precipitously developed hepatic enzyme abnormalities which promptly cleared on withdrawal of the drug. Biopsy of his liver revealed lymphoid infiltrates in portal areas with modest mast cell infiltrates. This patient also sustained granulocytopenia 1-1/2 years ago on aspirin with loss of bone marrow granulocyte precursors. Bone marrow biopsies were performed before resuming aspirin and at the time aspirin and at the time aspirin was terminated due to liver toxicity. These tissues are being processed for electron microscopy.

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* Salaried by Immunology Branch, NCI, NIH.

We have concentrated our efforts on the examination of the rheumatoid factor (RF) interaction with IgG. Because of findings of several laboratories, suggesting RF reacts with a conformational determinant of Fcγ generated during an antigen-antibody interaction, we have attempted to ascertain if that is indeed the case. Our general approach has been to examine IgG inhibition of the agglutination of Ripley anti D coated r_1, r_1 cells by RF. If the Ripley anti D antibody (IgG) undergoes a conformational change in the Fc region upon binding to erythrocytes, then denatured IgG should be much more potent than native IgG as an inhibitor of "Ripley cell" agglutination by RF. We have used pooled normal IgG and myeloma IgG in native and aggregated form as inhibitors. One of the IgG myelomas binds the DNP ligand with a Ka of 10^4 L/M and it was used with and without ligand as an inhibitor. The studies have shown no difference in patterns of inhibition in the presence or absence of ligand. Heat aggregated IgG is a much more effective inhibitor than an equal weight amount of native IgG. We feel that the most likely interpretation of these experiments is not that of Fc conformational change but that aggregated and hence multivalent IgG is bound more avidly than native-"univalent" IgG by RF. This can be explained by the energetic advantage of multivalent binding over univalent binding. However, recent experiments have indicated that some heated but not grossly aggregated myeloma IgG's are also more potent inhibitors than the native myeloma. Therefore, the possibility still exists that Fc conformational changes can lead to binding of greater avidity. We are isolating non-aggregated myeloma from heat aggregated preparations to critically test this point. Because of the low (10^4 L/M) Ka of the DNP binding myeloma, we feel these experiments are inconclusive.

Our interest in the nature of the interaction of IgG with RF is prompted by the following considerations. If RF preferentially binds a conformational determinant on Fc induced by the reaction of IgG antibody with antigen, then that IgG bound by RF _in vivo_ and isolatable from serum as part of an IgG-RF complex might well be antibody with specificity for antigens of importance in the pathogenesis of rheumatoid arthritis. We are testing this hypothesis in rabbits with RF's induced by hyperimmunization with heat killed _E. coli_. If the hypothesis is correct, IgG isolated from RF complexes should be almost entirely antibody to _E. coli_. We have been successful in producing RF by this technique and have isolated IgG from the complex. Experiments are underway to examine its specificity.

Significance to Biomedical Research and the Program of the Institute:

The significance of this research is discussed above. If our hypothesis is supported by the rabbit experiments, similar studies will be undertaken with human RF complexes.

Proposed Course:

The studies with patients and on the nature of the RF-IgG interaction are almost complete and will be prepared for publication. The remainder of the project will be terminated due to the departure of the principal investigator. The principal investigator intends to apply for extramural support for the continuation of these studies.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-26(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Porcine Immunoglobulins

Previous Serial Number: SAME

Principal Investigators: Dr. John S. Johnson

Other Investigators: NONE

Cooperating Units: Dr. John L. Decker, A & R, NIAMD (No Project No.)
Dr. Robert Ennis, A & R, NIAMD (No Project No.)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	4/12			4/12
Other:	0			0

Project Description:

Objectives:

Further characterization of porcine γA and study of immunoglobulin containing cells in the tissues of swine with chronic *Mycoplasma hyorrinus* arthritis.

Methods Employed:

Gel filtration and ion exchange chromatography. Standard zone electrophoresis techniques in agar and polyacrylamide; immunodiffusion; analytical ultracentrifugation; fluorescence microscopy.

Major Findings:

We have devised a method for separating 10S and 11S colostral IgA based on differential solubility characteristics. Because of the similarity of these two populations of IgA molecules to the canine serum and secretory IgA's,we feel they represent dimeric IgA with and without secretory piece. These immunoglobulins have been identified as IgA on the basis of immunological cross reactivity with human IgA. The molecular weights of their α and L chains have been determined by gel filtration in guanidine-HCl and are approximately 60,000 daltons and 23,000 daltons, respectively. Light chains are released from both molecules by gel filtration in strong dissociating solvent making it likely that both 10S and 11S IgA contain populations of molecules which are homologues of IgA_2 in other species.

Fluorescence microscopy of tissues from infected swine using previously developed class specific anti-heavy chain reagents have revealed immunoglobulin secreting cells of all 3 major classes in synovium. They are found almost exclusively in perivascular locations suggesting response to blood borne antigens. Significantly, there is no evidence of precipitated complexes in the vessel walls.

Significance to Biomedical Research and the Program of the Institute:

The study of immune paremeters in these swine has been pursued because of characteristics similar to rheumatoid arthritis in humans.

Proposed Course:

The present studies will be completed within the next month. This project will not be renewed because of the departure of the principal investigator.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-27(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Host Defense Mechanisms

Previous Serial Number: SAME

Principal Investigator: Dr. John N. Sheagren

Other Investigators: Dr. Harvey Simon

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2-6/12			2-6/12
Professional:	2-6/12			2-6/12
Other:	0			0

Project Description:

Objectives:

1) To study basic mechanisms involved in the acquisition of cellular immunity by macrophages and to elucidate the bactericidal mechanism in such cells.

2) To study the clinical and immunologic aspects of patients with sarcoidosis and to evaluate their response to therapy.

Methods Employed:

Studies of cellular immune function use peritoneal exudate cells (lymphocytes and macrophages) from strain II guinea pigs. The cells are placed in short term tissue culture and their bactericidal capacity quantitated under various experimental conditions by lysing the cells and doing serial pour plates and colony counts.

Patients: On clinical service of NIAID.

Major Findings:

1) Macrophages from guinea pigs immune to bovine gamma globulin (BGG) show enhanced ability to kill a typical facultative intracellular parasite, Listeria monocytogenes, when cultured overnite in the presence

of BCG. This enhanced bactericidal capacity can be demonstrated with any protein antigen administered so as to produce delayed hypersensitivity. The *in vitro* enhancement of macrophage killing capacity can be transferred to normal macrophages with purified immune lymphocytes.

2) Patients with sarcoidosis respond well to the initiation of prednisone therapy from the outset on an alternate-day dosage schedule. Steroid side effects are reduced.

Significance to Biomedical Research and the Program of the Institute:

1) Immunity to infections with facultative intracellular parasites (e.g. tuberculosis, brucella, deep mycotic infections and many parasitic and probably viral infections) seems to be mediated primarily through the macrophage. Our findings to date further relate the phenomenon of acquired cellular immunity to delayed hypersensitivity, and our *in vitro* system will enable its mechanism to be thoroughly elucidated.

2) The observation that sarcoidosis may be treated from the onset with alternate-day prednisone will result in decreased steroid side effects to patients with the disease and may extend the use of steroid therapy in sarcoidosis.

Proposed Course:

1) The mechanism of the development of the immunologically mediated enhancement of macrophage bactericidal capacity which we have described will continue to be explored. Emphasis will be on clarifying the interaction between antigen, the immune lymphocyte and the macrophage. Also, the metabolic aspects of the enhanced bactericidal capacity of the stimulated macrophages will be explored.

2) The sarcoid project will be terminated.

Honors and Awards: NONE

Publications:

1. Neva, F. A., Sheagren, J. N., Shulman, N. R., and Canfield, C: Malaria: Host-defense mechanisms and complications. *Ann Intern. Med.* 73:295-306, 1970.

2. Sheagren, J. N., Barth, R. F., Edelin, J. B., and Malmgren, R. A.: Mechanism of reticuloendothelial system blockade produced by anti-lymphocyte serum. *J. Immun.* 105:634-641, 1970.

3. Sheagren, J. N., Edelin, J. B., Barth, R. F., and Malmgren, R. A.: Antilymphocyte serum blockade of the reticuloendothelial system (an annotation). Am. Heart J. 81:144, 1971.

4. Simon, H. B. and Sheagren, J. N.: Cellular Immunity *in vitro* I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. Exp. Med. In press.

Serial No. NIAID-28(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Inflammation

Previous Serial Number: SAME

Principal Investigator: Dr. Harry R. Kimball

Other Investigators: Dr. Robert A. Clark
Dr. Burton C. West
Dr. Sheldon M. Wolff
Dr. David C. Dale
Dr. Alan S. Rosenthal
Dr. Michael M. Frank
Dr. Richard K. Root

Cooperating Units: Dr. John Decker, NIAMD (NIAMD-ARB-10c--project no.)
Dr. Marshall Lichtman, Rochester School of Medicine, Rochester, N. Y.
Dr. John Marshall, UCLA Medical Center, Los Angeles, California
Dr. Kenneth Melmon, University of California, San Francisco, California
Dr. George Padgett, Washington State University, Pullman, Washington

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	3-8/12			3-8/12
Professional:	3-8/12			3-8/12
Other:	0			0

Project Description:

Objectives:

1) Study the activity and distribution of lysosomal acid hydrolases in normal granulocytes and in the leukocytes of patients with Chediak-Higashi syndrome (CHS) and other leukocyte disorders.

2) Study of lysosomal enzymes in lymphocytes, monocytes, eosinophils and activated macrophages in both man and animals and correlate with specific functions.

3) Separate and identify different classes of granulocyte granules (lysosomes) of both normal and abnormal leukocytes and evaluate their biochemical and morphologic characteristics.

4) Determination of the effects of steroids, estrogens, chloroquine, bacterial products, antigen-antibody complexes on intact granulocyte lysosomes and their surrounding membrane.

5) Determination of intracellular distribution and function of lysosomal contents of normal and CHS leukocytes during phagocytosis and lymphocyte transformation and correlate with morphologic characteristics.

6) Study of bactericidal factors (phagocytin) and pyrogenic factors in normal and abnormal leukocytes.

7) Determine the _in vivo_ inflammatory properties of lysosomal products and establish their role in the inflammatory process.

8) Study of _in vitro_ leukocyte migration (chemotaxis) of normal and CHS leukocytes as well as granulocytes from patients with recurrent infections and correlate with _in vivo_ inflammatory responses.

9) Study leukocyte migration in altered metabolic states of uremia, acidosis, hepatic coma.

10) Study of origin, nature and adequacy of serum chemotactic factors in normals and patients with compromised host defenses.

11) Evaluate generation of serum chemotactic factors in hypocomplementemic animals (C4 deficient guinea pigs) and man (SLE).

12) Study _in vitro_ and _in vivo_ chemotaxis in CHS mink, cattle and beige mice (animal model of human CHS).

13) Identification and study of chemotactic factors in normal and abnormal human leukocyte lysosomal granules.

14) Evaluate resistance of beige mouse to fungal and bacterial infections.

15) Study of serum and marrow levels of lysozyme and establish its role as an indicator of granulocyte turnover and production in normal and leukopenic man as in cyclic neutropenia of dogs.

16) Study the vasoactive peptides ("kinins") in the inflammatory response to certain pyrogens.

17) Investigate the effects of estrogen and androgen on etiocholanolone fever.

18) Study of nonspecific resistance to fungal infections induced by bacterial endotoxins.

Methods Employed:

Peripheral blood leukocytes are separated into granulocyte and mononuclear fractions by gradient centrifugation in a Hypaque-Ficoll solution. Granulocytes are washed, lysed in heparin and sucrose to achieve full breakage of intact cells. Differential centrifugation is utilized to obtain granule rich fractions which are then studied for biochemical and morphologic (phase and EM) characteristics. Assays of the lysosomal enzymes, acid phosphatase (para nitrophenyl phosphate and beta glycerophosphate substrates), alkaline phosphatase, beta glucuronidase, lysozyme, myeloperoxidase, cathepsins are used as lysosomal markers. Assays of protein and non-lysosomal malic dehydrogenase are performed. Granule fractions are further separated by zonal or isopynic density gradient centrifugation into their various classes and studied for their biochemical and morphologic properties. Isolated leukocyte granules are incubated with various stabilizing and labilizing agents, separated by high speed centrifugation, and release of enzymatic content measured in the supernatant fractions.

Rebuck skin windows are utilized to measure leukocyte migration in man. A modification of the Boyden chamber technique is used to study chemotaxis *in vitro*. Dextran sedimented leukocytes are prepared for the chemotactic chamber and chemotactic factors are generated by incubating human or guinea pig serum with bacterial filtrates, endotoxins or antigen-antibody complexes.

Patients and volunteers are given adrenal steroids, estrogens and graded doses of etiocholanolone. Precise temperature responses are recorded and blood is obtained for measurement of marrow granulocyte reserves and plasma kinin levels. The latter is assayed using the rat uterus technique.

Mice are given endotoxin and subsequently challenged with *C. albicans*. Whole sera or fractions are passively transferred to normal mice to demonstrate a humoral factor in nonspecific resistance. RES clearance is performed utilizing aggregated I^{131} albumin. *In vitro* and *in vivo* growth of *C. albicans* is measured using homogenized tissue of treated animals or whole organ culture.

Major Findings:

1) Reproducible methods have been further developed for the separation of pure neutrophils and their granules from human blood.

2) Polyanions such as heparin are necessary for separation of intact granules. Heparin inhibits the action of several enzymes but can be reversed by protamine.

3) Normal human neutrophil granules are clearly separable into two and perhaps three or more biochemically and morphologically distinct populations. These conform to primary, secondary and tertiary granules classes identified in animal leukocytes.

4) Giant CHS granules have lowered activities of myeloperoxidase (25% of normal), beta glucuronidase (45% of normal), PNP acid phosphatase (60% of normal) and lysozyme (80% of normal). Cathepsin D and BGP acid phosphatase levels are normal while alkaline phosphatase is 2-fold higher. The intracellular distribution of these enzymes differs from normal.

5) Phagocytizing CHS leukocytes fail to release their lysosomal contents normally either to the extracellular media or to the intracellular phagocytic vacuole.

6) CHS leukocytes do not respond to chemotactic factors in a normal fashion (reduced to approximately 70% of normal), and one patient with CHS was found to have a marked deficiency in generating serum chemotactic factors.

7) A defect in cellular chemotaxis documented in CHS mink (animal model of CHS) which closely parallels that seen in CHS humans. This offers an animal model for the study of normal and abnormal cell migration.

8) A cell defect in chemotaxis has been identified in a child with recurrent and unexplained infections.

9) Serum generation of chemotactic factors is reduced in uremia and certain patients with SLE (unrelated to azotemia).

10) Free "kinin" levels are increased in the plasma of patients with known endotoxemia.

11) Serum muramidase parallels the production of marrow granulocytes both in man and dogs with cyclic neutropenia.

Significance to Biomedical Research and the Program of the Institute:

Evidence is accumulating that leukocyte lysosomes and their enzymes play an important part in the pathogenesis of certain inflammatory diseases. Most of this work has been done in experimental animals and conclusions inferred to the human pathologic state. Our purpose is to study human lysosomes, primarily from leukocytes, and establish their importance in both normal and abnormal inflammatory processes. The CHS is of special interest to us since this is a disease in which lysosomal mechanisms do not function properly. Defective migration of these cells to an inflammatory site in concert with lower activities of certain bactericidal enzymes and a faulty intracellular delivery system has major consequences for host resistance in these patients. Detailed knowledge of the biochemical nature of these granules and their contribution to the varied function of granulocyte will further clarify the nature of the CHS defect and increase our understanding of normal leukocyte function in the inflammatory process.

Proposed Course:

It is planned to continue the studies outlined above.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-30(c)
1. LCI
2. Biochemistry and Immunochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Interaction of Antibody in Complement in the Production of Immune Damage

Previous Serial Number: SAME

Principal Investigator: Dr. Michael Frank

Other Investigators: Dr. Joseph May
Dr. Michael Kane
Miss Thelma Gaither
Mr. Clinton Hoffler

Cooperating Units: Dr. Leonard Ellman, LI, NIAID-142
Dr. Ira Green, LI, NIAID-142

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2 2/12			2 2/12
Professional:	1 8/12			1 8/12
Other:	6/12			6/12

Project Description:

Objectives:

1. To determine the role that each of the various immunoglobulin classes play in complement mediated cytotoxic reactions, complement fixation and generation of biologically active products from complement components.

2. To evaluate the role of complement in immunologic diseases.

Method Employed:

Quantitative complement component assay, preparation of intermediates in the complement sequence with various complement components on the surface of cells and purification of complement components and antibodies of the various classes are routinely used in this laboratory. Specifically, these methods employ the use of DEAE cellulose and C-M cellulose, Sephadex, electrophoretic techniques and ultracentrifugation for the purification of immunoglobulins and complement component proteins. Cellular intermediates in the complement sequence (i.e. cells sensitized with antibody and reacted with various components of complement) are prepared by our own modification of standard methods and are used for titration of complement components. Efforts are made to determine the interrelationships and efficiency of various complement components reacting with other components from the same species and cross species lines. Purified preparations of various antibodies, antigens and endotoxins are used to study immunologic responsiveness of a new strain of guinea pigs recently established in our laboratory with a total deficiency of C4, one of the early reacting components in the complement sequence. Breeding experiments to establish and extend this line of animals are in progress.

Major Findings:

In last years annual report brief mention was made of the finding of guinea pigs with a total deficiency of C4. C4 represents the second complement protein in a series of nine proteins capable of interacting with antigen antibody complexes and destroying cells and tissues. Breeding experiments have shown that the deficiency of C4 is under genetic control and is complete. This new strain represents the first deficiency in any animals of a complement component which acts early in the complement sequence and is the only available genetic deficiency of any component in the guinea pig. These animals therefore present a major opportunity to discover the role of complement in the body's defenses and the function of complement in disease. Our major findings are as follows: Many of the inflammatory responses which are classically thought to be complement dependant are found to be intact in this new strain of guinea pigs with a genetically controlled total deficiency of C4. The animals have an intact Arthus reaction and intact passive cutaneous anaphylaxis reactions. They have a mild defect in their antibody response to several antigens, but this is not universally true for all antigens. They manifest normal delayed hypersensitivity to several antigens. The animals have a marked defect in the immune clearance of antibody coated, chromated red blood cells. A series of in vitro studies have therefore been performed to determine why these animals are capable of showing many complement mediated reactions in the total absence of C4. These studies indicate that the C4 deficient animals have a well developed by-pass mechanism to by-pass the early components of the complement sequence. Thus, they do not utilize C1, C4 and C2 to produce many inflammatory reactions. Use of their serum allows for the clear demonstration of this new pathway which has been postulated to exist in normal serum. These studies provide the first conclusive evidence for the existence of such a pathway and the first demonstration of its operation in vivo.

Significance to Biomedical Research and the Program of the Institute:

The pathway of immune damage which proceeds through the activation of the complement system of proteins is now felt to be involved in the etiology of many of the diseases which have an immunologic basis. Moreover, this same pathway is thought to be very important in normal defense mechanisms against tumor cells invading bacteria, etc. Our recent studies in the development of the new strain of guinea pigs with the total deficiency of C4 allows one to examine the role of complement in several of these situations. These studies show that the early components of complement are especially important in the clearance of red blood cells as occurs in patients with autoimmune hemolytic anemia. Nevertheless, a by-pass mechanism exists in the serum of these animals which can by-pass the early components of complement and enter the complement sequence of C3. This by-pass pathway allows for bacetericidal reactions, phagocytosis of bacteria, ect., in the absence of early complement pathway. This alternate pathway had been postulated but had never been unequivocally demonstrated. These studies will redirect research in many laboratories toward exploration of this alternate mechanism for activation of the late complement components for further definition of their importance in human clinical disease states.

Proposed Course:

These studies will be continued in an attempt to find this alternate pathway in greater detail.

Honors and Awards: NONE

Publications:

1. Frank, M. M. and Gaither, T.: The effect of temperature on the reactivity of guinea-pig complement with γG and γM haemolytic antibodies. Immunology 19: 967-974, 1970.

2. Bragonier, J. R., Frank, M. M., and Brent, R. L.: Production of congenital malformation using tissue antisera. J. Immun. 105: 1175-1180, 1970.

3. Frank, M. M. and Gaither, T.: Evidence that rabbit γG haemolysin is capable of utlizing guinea-pig complement more efficiently than rabbit γM haemolysin. Immunology 19: 975-981, 1970.

4. Fauci, A. S., Frank, M. M., and Johnson, J. S.: The relationship between antibody affinity and the efficiency of complement fixation. J. Immun. 105: 215-220, 1970.

5. Frank, M. M.: Physiology of the immune response. In Rhyne, M. (ed.): Hay Fever in Childhood. p. 41.

6. Frank, M. M., Dourmashkin, R. R., and Humphrey, J. H.: Observations on the mechanism of immune hemolysis: importance of immunoglobulin class and source of complement on the extent of damage. J. Immun. 104: 1502-1510, 1970.

7. Frank, M. M. and Gaither, T. A.: Complement fixation by a single molecule of γG hemolysin. J. Immun. 104: 1458-1466, 1970.

8. Reviewed by Dr. Michael Frank: Book Review on A new, genetically controlled complemtn abnormality: C4 deficiency in the guinea pig. Reviewed in Proceeding of the International Symposium on Complement. In press.

Serial No. NIAID-33(c)
1. LCI
2. Clinical Allergy and Hypersensitivity Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathogenesis of Delayed Hypersensitivity

Previous Serial Number: SAME

Principal Investigator: Dr. Charles H. Kirkpatrick

Other Investigators: Dr. Robert Rich
Dr. Richard Johnson
Dr. Alan S. Rosenthal
Dr. David Dale

Cooperating Units: Dr. Harold Baer, DBS-102
Dr. Ira Green, LI, NIAID-142
Dr. Arthur Schade, LM, NIAID-63c
Dr. Louis Diamond, LPD, NIAID 125-K
Mr. Milford Lunde, LPD, NIAID-125-P

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	3 8/12			3 8/12
Professional:	3 8/12			3 8/12
Other:	0			0

Project Description:

Objectives (Sub-project A):

1. To characterize the method of action of phytohemagglutinin (PHA) on human lymphocytes.

2. To determine the effect of submitogenic doses of PHA on antigen induced lymphocyte transformation.

3. To extend the above investigations to lymphocytes from patients suspected of having defects in cellular immunity.

Method Employed:

Peripheral blood lymphocytes from healthy human volunteers and patients with chronic mucocutaneous candidiasis were cultured with different concentrations of PHA. The threshold doses, 50% maximal response doses, peak response dose and slope of the linear ascending limb of the dose-response curves were computed. The effects of autologous, homologous and heterologous sera on the response curves were also studied. The response was quantitated by measuring incorporation of tritiated thymidine into DNA.

Major Findings:

Antigen-induced DNA synthesis was greatly enhanced by addition of sub-mitogenic dose of PHA. The amplitude of these responses were more than additive and were compatible with synergy between non-specific mitogens and specific antigen leading to enhanced DNA synthesis.

In patients with chronic mucocutaneous candidiasis the maximal and 50% maximal DNA synthetic responses were observed at the same doses as control subjects. However, the sope of the linear portion of the dose response curve of the candidiasis patients was considerably less than the controls. This finding is compatible with either a smaller number of responding cells or receptor sites, or an impairment in the cell interactions required for the antigen-induced DNA response. At present we have not been able to resolve this question, but it continues to be investigated.

Proposed Course:

These studies will be extended to include additional subjects and to investigate the possible role of serum factors in the aberration of the PHA response.

Significance to Biomedical Research and the Program of the Institute:

These studies provide indirect evidence for biological "receptors" for PHA and antigens onlymphoid cells. Enhancement of the antigen-induced DNA synthesis by small doses of PHA could occur through several mechanisms. Cells may possess different receptors that are specific for antigen or mitogen, but only a certain number of these receptors need to be occupied for initiation of DNA synthesis. The PHA could function as a non-specific amplifier by filling some of the crucial receptors. Experiments with varying doses of antigens and mitogens are planned to evaluate this possibility, and further define the nature and properties of these receptors.

Experiments described in sub-project B also deal with patients with chronic mucocutaneous candidiasis. The studies with PHA suggest that the lymphocyte defect is rather extensive, and that while most striking with the specific response to Candida albicans, it also involves the relatively less specific responses to mitogens such as PHA.

Objectives (Sub-project B):

1. Definition of the immunological abnormalities in chronic mucocutaneous candidiasis.

2. Evaluation of immunologic reconstitution in the management of candidiasis.

Method Employed:

Eleven patients with chronic candidiasis have been admitted to the Clinical Center facilties of LCI and three subjects studied elsewhere bring our total experience to 14.

Each subject has had detailed studies of cellular and humoral immunologic responses, leukocyte phagocytic capacity and leukocyte enzymology and endocrine responses. Specifically, the evaluation included in vivo ascessment of delayed hypersensitivity with a panel of antigens, and in vitro studies of lymphocyte function such as antigen induced DNA synthesis and production of macrophage migration inhibition factor (MIF).

Major Findings:

Ten of the subjects with chronic mucocutaneous candidiasis had clinical evidence of impairment of cellular immunity to C. albicans. In three subjects this defect involved all antigens studied, and these patients could not be sensitized with chloro-dinitrobenzene. In seven subjects the defect was limited to the antigens of C. albicans. In every case, the serological responses have been intact.

In vitro studies of lymphocyte functions provided addition information regarding the nature of the cellular immune defect. Of the ten subjects with negative delayed cutaneous reactions to C. albicans antigen, eight also failed to show in vitro proliferative responses when their lymphocytes were exposed to candida antigens. Two had normal responses. However, none of the subjects studied produced MIF, a substance generally suspected of being a "mediator" in the pathogenesis of delayed hypersensitivity.

Restoration of the immune response has been attempted in five patients. Two patients received massive transfusions of allogenic immunocompetent lymphoid cells. In one patient, the previously negative skin tests promptly became positive and remained positive for at least 220 days. Within 3 weeks there was striking clearing of the lesions. However, approximately 10 months later new candida lesions appeared and when his immune responses were studied, the previously positive in vivo and and in vitro responses had become negative. The second patient had no in vivo and in vitro evidence of correction of the immune defect. Clinically, however, there had been vast improvement with no active mucosal infections with Candida since the transfusions.

Because of the potential adverse affects of allogeneic cells, we do not plan to pursue this approach. Transfusions of viable cells will be used only when genetically matched donor is available.

Two patients have received "transfer factor", a dialyzable substance from lysed immune lymphocytes. In both cases, there has been in vivo and in vitro evidence of restoration of cellular immunity. It is too early to ascess clinical responses.

Significance to Biomedical Research and the Program of the Institute:

These findings suggest that certain disorders such as chronic fungal infections may result from impaired cellular immunity. Whether the defect occurs as an inborn error or is acquired (tolerance) is not known, but is under study. At present the findings are interpreted as indicative of two types of lymphocyte defects in chronic mucocutaneous candidiasis. Patients who fail to produce MIF and fail to respond to antigenic stimulation with increased DNA synthesis are believed to lack the antigen-reactive cells required for initiation of cellular responses to candida antigens. Patients with normal DNA synthesic responses but no MIF production are believed to possess antigen-sensitive cells, but are deficient in either the mediator producing cells or the differentiative steps required for an antigen-sensitive cell to produce and release mediators. Considering that humoral antibody synthesis is normal, this selective defect in cellular responses is reminicent of "immune deviation" in animals. Therefore, chronic candidiasis, appears to provide a model for evaluation of the therapeutic potential of immunologic reconstitution in humans, as well as study of the role of cellular immunity in resistance to other disorders including chronic infections and neoplastic diseases.

Proposed Course:

The future studies will follow two general themes: 1) definition of the defect in cellular immunity, and 2) development of therapeutic techniques. The plaque assay technique for detecting antigen-reactive cells is being established to characterize the number of these cells in candidiasis patients. This should distinguish between two possible defects - absence of antigen-reactive cells or a defect in differentiation of reactive cells to mediator-producing cells.

Transfer factor with and without amphotericin is being evaluated in correction of immune defects in patients with candidiasis.

Objectives (Sub-project C):

1. Evaluation of agents that compete for growth factors required by *Candida albicans*.

Method Employed:

Two iron-binding substances, lactoferrin and deferroximine were studied. Various concentrations of the chelating agents were added to suspensions of *Candida albicans* and the effects on colony numbers and colony size were determined.

Using a radial diffusion assay with specific anti-lactoferrin antisera, the concentrations of lactoferrin in the parotid fluids of patients with chronic mucocutaneous candidiasis and normal control subjects were measured.

A fluorescent antibody was used to determine the presence and location of lactoferrin in leukocytes from normal subjects as well as patients with chronic mucocutaneous candidiasis and other chronic infections.

Major Findings:

Chelating agents were effective in retarding growth of C. albicans and this effect was lost when the chelator was saturated with iron. Lactoferrin concentrations in the parotid fluids of candidiasis patients were not different from control subjects.

Lactoferrin was found to be present only in mature polymorphonuclear leukocytes and band forms, but not in other cells in the peripheral blood or bone marrow. In contrast to a previous study, the lactoferrin was limited to the nuclear membrane rather than throughout the cytoplasm. This was supported by the finding that lactoferrin could be detected in eluates from isolated nuclei. The distribution of lactoferrin in cells from patients with chronic infections was not different from the controls.

Significance to Biomedical Research and the Program of the Institute:

Infectious diseases occur when a pathogenic organism invades a normal subject, or when opportunistic organisms enter tissue of subjects with impaired defense mechanisms. These studies were undertaken to examine the possibility that patients with chronic candidiasis and other chronic infections may have impaired capacity to chelate iron, an essential growth factor for many organisms, as a component of their impairment. No evidence for such a defect was found. The marked inhibition of growth of C. albicans by chelating agents suggested that these agents may be useful in topical treatment of superficial candidiasis.

Proposed Course:

At present there are no plans to extend these experiments.

Objectives (Sub-project D):

1. Identification of the antigen-sensitive cell responsible for this graft-vs-host reaction (GVH).

2. Development of method of selectively removing the GVH cells.

Method Employed:

Spleen cells from rats that differ at major histocompatibility loci (AgB) will induce GVH when given to neonatal or irradiated adult recipients. Similar reactions may be produced with bone marrow cells but this may reflect contamination with peripheral blood lymphocytes.

Three methods have been employed to selectively remove GVH cells from spleen cell suspensions. Glass beads coated with cells from one strain of rat (A) are poured into a column. Spleen cells from strain (B) that differs from strain A at AgB are passed over the column and the effluent cells are examined for their ability to produce GVH and positive mixed lymphocyte reactions (MLR). In principle, the cells from strain B that have recognition sites for A should be retained or retarded on the column. An alternative method employes the same principle of cell recognition, but depends on the ability of allogeneic cells to form aggregates or rosettes in vitro. The rosettes of antigen-sensitive cells are removed by differential centrifugation and the supernatant cells have been studied in the GVH and MLR systems. Thirdly, lymphoid cells that have been stimulated to divide by exposure to allogeneic histocompatibility antigens then treated with bromdeoxyuridine (BUdR) and ultra-violet light have been studied in GVH and MLR assays. In theory, the cells that are selectively stimulated to divide will be killed by BUdR and light and will be unable to produce GVH or MLR.

Major Findings:

Both the antigen coated glass bead columns and the mixed cell suspensions can remove cells that produce MLR. The method still has some variability and development of a consistently reproducible system constitutes a major effort at present. The conditions of coating the beads is crucial to obtaining viable cell effluents. Similarly, the use of protein gradients to improve separation of cells by centrifugation is under study. BUdR-light treatment appears to be the most effective method for deleting specific antigen-reactive cells from a mixed population. Marked reduction of GVH and MLR have been obtained by this method.

Significance to Biomedical Research and the Program of the Institute:

Treatment of certain diseases by transplantation of immunologically or hematopoietically competent cells constitutes a new dimension in management of disorders that until recently were iniformly fatal. For example, children with the lymphopenic form of agammaglobulinemia and the Wiskott-Aldrich syndrome have been reconstituted with bone marrow grafts, and the technique is currently under study in patients with drug resistent leukemia. A major obstacle in this area has been the frequent occurrence of fatal graft-vs-host reactions.

The studies described above are directed at devising methods for selectively removing cells that cause GVH but leaving cells that would reconstitute the hematopoietic and lymphopoietic functions.

Proposed Course:

This project is primarily the responsibility of an Associate and will presumably leave with him in June, 1971.

Objectives (Sub-project E):

1. Photosensitive chelitis.

Methods Employed:

A patient referred for study of chronic candidiasis was found to have a different disease. For two years, he had noted painful, chronic ulceration of his lower lip, which after considerable study was proved to be related to exposure to sunlight. Histology of light-exposed and light-protected areas was done. DNA repair was also measured following ultra-violet injury.

Histologic studies showed unique findings. Sun-exposed areas became ulcerated and cells in the ulcers contained bizarre nuclear bodies. After healing, the cells contained fewer nuclear bodies. Electron micrographs also disclosed numerous microtubules similar to those reported in glomerular basement membranes in SLE, and believed to be of viral etiology.

The effects of ultra-violet light on DNA synthesis was measured by studying tritiated thymidine uptake by hydroxyurea-blocked cells. The results of this study were normal, excluding a defect in DNA repair as the basis of the chronic disorder.

Significance to Biomedical Research and the Program of the Institute:

Establishment of the basis of his chronic chelitis led to a program of successful treatment.

Proposed Course:

This project has been completed.

Objectives (Sub-project F):

To evaluate an antigenic extract of Entameba histolytica in normal subjects.

Methods Employed:

Normal volunteers without evidence of amebiasis were skin-tested twice with histolyticin from Entameba histolytica. Serial sera were collected and assayed for antibody activity.

Major Findings:

Of 23 subjects tested twice with 2 μg of histolyticin, one had a positive response 24 hours following the second test. None of the eight subjects tested twice with 4 μg had positive responses.

When the dose was increased to 8 μg, 3 of 10 volunteers had positive reactions 15 minutes after the first test. Of greater interest were the results of the second skin tests. Six of 10 subjects had positive reactions at 15 minuts and 4 of 10 subjects reacted at 24 hours. No serological responses were detected.

Significance to Biomedical Research and the Program of the Institute:

These findings indicate that an initial exposure of a normal subject to 8 μg of histolyticin alters his cutaneous response to a second test. The 8 μg dose, therefore, appears excessive for routine diagnostic or epidemiologic studies. This finding is especially important for investigators working in endemic areas such as the group involved in PL-480 project 01-066-1 in New Delhi.

The nature of the 24 hour reactions is unknown, but they are tentatively interpreted as Jones-Mote reactions rather than delayed hypersensitivity.

Proposed Course:

Two lines of investigation remain: 1) establishment of the proper dosages of antigen for routine clinical studies, and 2) characterization of the nature of the cutaneous responses. The first study presumably will be conducted in India under a PL-480 contract. The second will probably not be done in humans because of ethical considerations. Although not planned, the question could probably be answered with rats.

Objectives (Sub-project G):

Study of cutaneous reactions of patients with poison ivy to catechols from Rhus radicans.

Methods Employed:

Twenty-six patients with histories of mild to moderate contact reactions to poison ivy were studied. Each patient received patch tests with 10^{-2}, 10^{-3}, and 10^{-4} M pentadecyl catechol (PDC) and three related, but unsaturated substances, 8 -dehydro PDC (mono), 8 , 11 - dehydro PDC (Di), 8 , 11 , 14 -dehydro PDC (Tri). Cutaneous responses were measured on day 2, 3, 5 and 7.

Major Findings:

The Di- and Tri- unsaturated derivatives were found to be the most antigenic. Reactions to these compounds occurred with lower concentrations of antigen and appeared earlier and were positive in a higher percentage of subjects. In contrast reactions to PDC were uncommon and responses to the mono-derivative occurred in about half the subjects.

Significance to Biomedical Research and the Program of the Institute:

The susceptibility to sensitization with antigens from Rhus radicans is believed to be inherited, and two general proposals for treatment have been offered. One involves feeding newborn offspring the antigens in an attempt to induce tolerance or a related form of unresponsiveness, and the second involves suppression of established allergy by "desensitization". In either case, identification of the major sensitizing agents is a necessary requisite. This study provides evidence of the differential antigenicity of the catechols from poison ivy.

Proposed Course:

This project has been completed.

Honors and Awards:

Photosensitive Cheilitis - For presentation at National Meeting, American Federation for Clinical Research, May, 1971.

Publications:

1. Dr. Charles H. Kirkpatrick, reviewer, Book Review on Biology of the Immune Response, In: Abramoff, P. and La Via, M. F. (eds.): Reviewed in Ann. Intern. Med. 74: 461, 1971.

2. Kirkpatrick, C. H.: Immunosuppressive therapy. Postgrad. Med. 47: 151-157, 1970.

3. Kirkpatrick, C. H., Stites, D. P., Smith, T. K., and Johnson, R. A.: A factor which enhances DNA synthesis in cultures of stimulated lymphocytes. In: Fourth Leukocyte Culture Conference.

4. Kirkpatrick, C. H., Chandler, J. W., Jr., Smith, T. K., and Newberry, M. W., Jr.: Cellular immunologic studies in hstioplasmosis. In: Second National Conference on Histoplasmosis. In press.

5. Kirkpatrick, C. H., Rich, R. R., Graw, R. G., Jr., Smith, T. K., Mickenberg, I. D., and Rogentine, G. N.: Treatment of mucocutaneous moniliasis by immunologic reconstitution. Clin. Exp. Immun. In press.

6. Kirkpatrick, C. H., Rich, R. R., and Bennett, J. E.: Chronic mucocutaneous candidiasis: Model building in cellular immunity. Ann. Intern. Med. In press.

7. Green, I., Kirkpatrick, C. H., and Dale, D. C.: Lactoferrin: Specific localization in the nuclei of human polymorphonuclear neutrophilic leukocytes. Proc. Soc. Exp. Biol. Med. In press.

Serial No. NIAID-36(c)
1. LCI
2. Electron Microscope Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Structural Correlates of Antigen and Mitogen Interactions with Immunocompetent Cells

Previous Serial Number: NONE

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigator: Dr. D. L. Rosenstreich
Mr. J. Thomas Blake
Dr. J. Johnson

Cooperating Units: Dr. J. M. Davie, LI, NIAID, NIH
Dr. William Paul, LI, NIAID, NIH
Dr. Jack Stobo, LI, NIAID, NIH

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1			1
Professional:	1			1
Other:	0			0

Project Description:

Objectives:

1) To develop general and specific cytochemical techniques which would permit visualization of antigen at the light and electron microscope level. Electrophoretically pure horseradish peroxidase and related peroxidases have been chosen for study because they will potentially permit both light and electron microscope study.

2) To correlate at a morphologic level cell interaction and the immunologic responses of such a model system.

3) To determine the cellular fate of antigen or mitogenic proteins in *in vitro* and *in vivo* models of cellular and humoral immunity using visible markers.

Methods Employed:

Electrophoretically pure horseradish peroxidase and cytochrome C peroxidase have been chosen as antigens with potential as visible immunologic markers. The following techniques have been applied to permit characterization of the immunobiology of these proteins as antigens and record the factors determining their use as detectable antigens at a cytochemical level:

1) Deoxyribonucleic acid synthetic response of lymph node, spleen, thymus and peripheral blood cells.

2) Nature and characterization of the antibody synthetic response _in vitro_ and _in vivo_. A technique for simultaneous immunodiffusion studies and cytochemistry has been developed.

3) Isolation of anti-HRPO binding material from guinea pig serum by selective immune absorbent column chromatography and subsequent purification over Sepharose G-200 into Ig subclasses.

4) Specific antisera were raised against the Ig and anti-HRPO antibody and were characterized by Farr precipitation assays and hemagglutination studies performed by established and published techniques.

5) Preparation of antigenically active but enzymatically inactive derivatives are prepared by acid-acetone extraction of the heme group at -40°C, the heme moiety being required for enzymatic activity of the peroxidatic protein.

6) Horseradish peroxidase (HRPO) can be coupled to other immunogenic proteins by using gluteraldehyde as a cross-linking reagent at pH 7.0 and the derivative purified by starch block electrophoresis.

7) Antigen binding studies: Teased lymph node or spleen cells are washed x 3 in 1% serum-suspension media (Eagle's) and at a concentration of 100-200 million per milliliter incubated with or without antigen at various concentrations and at 4°C for 30-60 minutes. These cells are then washed 3-4 times over 100% fetal calf serum by density gradient and resuspended in 0.5 ml pure fetal calf serum and smeared on glass slides. Cells are air dried and fixed in either gluteraldehyde, methanol (-40°C) or acid-acetone (-40°C-70°C). After washing away fixative some slides are incubated with horseradish peroxidase and fixed in gluteraldehyde. Slides are then incubated in 3,3-diaminobenzydine in tris-HCl buffer at pH 7.6 and reaction product visualized after mordinification and dehydration for light microscopy.

Alternatively aliquots of gluteraldehyde fixed cells were processed for electron microscopy.

Major Findings:

1) Rabbits and guinea pigs have been immunized with horseradish peroxidase. Rabbits respond by formation of antibody, and a deoxyribonucleic acid synthetic response in cell cultures of lymph nodes, spleen and peripheral blood. Guinea pigs make little detectable precipitating antibody but develop cellular immunity to horseradish peroxidase.

2) Using cytochemical techniques at both the light and electron microscope level, lymph nodes from rabbits immunized to horseradish peroxidase (HRPO) can be shown to contain lymphocytes and plasma cells which synthesize antibody (5 cells/per 100) and bind enzymatically active HRPO. Control (Freund's Adjuvant immune) cells contain no "detectable" antibody forming cells. Guinea pigs which exhibit cellular immunity to HRPO and have no apparent precipitating immunoglobulin have no cytochemically positive cells.

3) A circulating material has been observed in guinea pigs immune to HRPO which inhibits the hemagglutination of HRPO coated sheep RBC and precipitation of HRPO with rabbit anti-HRPO Ig. This material appears to bind HRPO and in studies in collaboration with Dr. J. Johnson of the Laboratory of Clinical Investigation, we have characterized this material as a new Ig possessing unique antigenic specificity as demonstrated by immunochemical techniques.

4) Preliminary studies in the rabbit were begun on characterization of the role of antibody forming cells and their precursors in the *in vitro* proliferation response mediated by specific antigen. These studies indicate that antibody forming cells, in contrast to previous suggestions, proliferate *in vitro* as well as thymus derived cellular immune cells. Furthermore, cells actively engaged in antibody formation synthesize new DNA.

Significance to Biomedical Research and the Program of the Institute:

A knowledge of structural correlates of the cellular events in immunologic interactions is necessary to fully comprehend such basic phenomena as transplantation rejection and immunoglobulin synthesis. Such an understanding in these areas would contribute greatly to the likelihood of therapeutic and pharmacologic manipulation of crucial host defense mechanisms. Macrophage migration appears clearly to be an *in vitro* correlate of cellular immunity and as such provides a model for studies of the ultrastructure and cellular correlation of delayed hypersensitivity.

Proposed Course:

1) Attempts to synthesize HRPO conjugates with mitogen (Concanavalin A) with Drs. J. Stobo and W. Paul of the Laboratory of Immunology will begin. After initial characterization of the biochemical and immunologic properties of these compounds the localization of mitogen receptors end their fate in the *in vitro* proliferation response will be studied.

2) In collaboration with Drs. J. Davie and W. Paul of the Laboratory of Immunology, we will synthesize conjugates of rabbit anti-guinea pig immunoglobulin. Once synthesized the immunoglobulin membrane surface on guinea pig lymphocyte receptors will be mapped and characterized.

3) The contribution of antibody-forming cells and their precursors in antigen-mediated *in vitro* lymphocyte proliferation will be completed.

4) The fate of antigen binding cells using conjugates of HRPO and dinitrophenyl guinea pig albumin (DNP-GPA) or radioactive DNP-GPA will be characterized and their light and ultrastructure explored.

a) Determine the kinetics of the appearance of antigen binding cells (ABC) and antibody forming cells (AFC) and explore the localization of antigen at the level of the electron microscope.

b) Quantitation of receptors per cell and its relationship to the immunocompetence of the animal.

c) Attempt to differentiate antibody forming cells precursor from antibody binding cells and their origin (thymic or marrow).

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-37(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Synthesis of Cytochrome P-450 of the Liver

Previous Serial Number: SAME

Principal Investigator: Chull S. Song, M.D., LCI, NIAID

Other Investigators: Mrs. Nancy A. Gelb, LCI, NIAID
Alan S. Rosenthal, M.D., LCI, NIAID

Cooperating Units: Harold L. Moses, M.D., Department of Pathology,
Vanderbilt University, School of Medicine,
Nashville, Tenn.

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total	8/12			8/12
Professional:	2/12			2/12
Other:	6/12			6/12

Project Description:

Objectives:

1) To continue the study of mechanisms underlying the induction of microsomal cytochrome P-450 of the liver.

2) To correlate the biochemical and ultrastructural aspects of the drug induction of hepatic cytochrome P-450 and porphyrin and heme synthesis in developing rats.

Methods Employed:

Biochemical determinations of hepatic -aminolevulinate (ALA) synthetase, cytochrome P-450, cytochrome b_5, and porphyrin and heme contents are as described in the previous annual report.

Seven day old rats were separated from mothers and treated with allylisopropylacetamide or phenobarbital. They were sacrificed 16-20 hours later and various biochemical parameters were determined on their livers as described above. Samples of the livers were also fixed in glutaraldehyde for electron microscopic studies by Drs. Rosenthal and Moses.

Major Findings:

1) In contrast to the adult rat liver parenchymal cells which contain an abundance of smooth endoplasmic reticulum (E.R.), the liver cells of neonatal rats contain very little discernible smooth E.R. This new observation is in accord with our previous biochemical finding that the neonates contain substantially smaller amounts of hepatic cytochrome P-450, which is the terminal oxidase in the mixed function oxygenase system of the liver and which is present in the smooth E.R.

It also provides the ultrastructural confirmation of the long-known fact that the neonatal rats have very limited capacity to oxidize various drugs due to limited amounts of hepatic microsomal enzymes concerned with drug oxidations, of which the mixed function oxidase system is functionally the most important one.

2) Allylisopropylacetamide (AIA), a drug which increases in adult rats the hepatic synthesis of porphyrins, heme, and hemoproteins such as cytochrome P-450 by induction of the liver enzyme ALA synthetase, concomitantly induces marked morphologic changes in the liver cells. These changes include cellular hypertrophy, proliferation of smooth E.R. and production of lipoprotein granules. We have previously shown that AIA produced none of the biochemical alterations in the neonatal livers. We have now obtained an ultrastructural correlation to this finding. When AIA was given to neonatal rats in amounts equivalent to those which induced biochemical and morphological changes in adults and their livers were examined in the electron microscope, none of the ultrastructural alterations observed in the adult livers were seen. The neonatal livers were completely unchanged by the drug.

3) Phenobarbital, a drug which induces a marked proliferation of smooth E.R. in adult rat livers concomitant with increase in the amount of cytochrome P-450, does not produce a significant change in the amount of hepatic smooth E.R. in the neonatal rats. Although neonatal rats do respond to phenobarbital by increasing the hepatic content of cytochrome P-450 (and microsomal mixed function oxygenase activities), the absolute amount of this hemoprotein still is probably much less than that present in the adult liver. This, plus the diminished responsiveness of the neonatal liver to induction of other microsomal proteins and enzymes, probably account for the little ultrastructural alterations observed with phenobarbital.

Significance to Biomedical Research and the Program of the Institute:

Developmental changes in the biochemical pathway involved in the porphyrin and heme synthesis and the synthesis of the hemoprotein, cytochrome P-450, have been the object of the current series of studies. Drugs such as AIA and phenobarbital, which markedly alter this pathway in the adult rats and which produce highly specific alterations in the ultrastructural morphology of the liver cells, have little or no effect in the neonatal rats and produce no significant morphologic alterations in their liver cells. Thus, the limited amount of hepatic cytochrome P-450 and the limited extent of its induction in the neonatal livers correlate well with virtual absence of smooth E.R. in their liver cells. The absence of induction of hepatic ALA synthetase on treatment with AIA in the neonates correlates with the absence of any of the ultrastructural changes that AIA regularly induces in the adult livers. Thus, biochemical and morphological correlations have been made on the developmental aspect of the regulation of hepatic synthesis of a key enzyme concerned with drug metabolism.

Proposed Course:

To be discontinued.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-38(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Antigenic Competition

Previous Serial Number: NONE

Principal Investigator: Dr. John S. Johnson

Other Investigators: Dr. Anthony S. Fauci
Dr. Michael M. Frank
Dr. Richard Johnson

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1-7/12			1-7/12
Professional:	1-7/12			1-7/12
Other:	0			0

Project Description:

Objectives:

Elucidation of the mechanism whereby simultaneous immunization with 2 or more immunogens result in suppression of the immune response to one or all of the immunogens (antigenic competition).

Methods Employed:

Gel filtration, ion exchange and affinity chromatography; immunodiffusion analyses; localized hemolysis in gel assay of antibody secretory cells (Jerne plaque technique); standard complement fixation techniques; determination of binding energies by hapten quenching of tryptohane fluorescence.

Major Findings:

Further studies have indicated that non-crossreacting haptens on non-crossreacting carriers also exhibit "competition" whether given in the same or distant sites. We have also found that preimmunizing the animals with carrier so as to augment carrier specific ARC does not abolish competition but rather augments it. These findings together with those discussed in last year's annual report are not compatible with competition and indicate the presence of a circulating inhibitor produced concomitantly with antibody synthesis. The inhibitor is not antibody. In general, the inhibitory activity has paralleled the magnitude of the immune response. In the course of these studies we have utilized keyhole limpet hemocyanin (KLH) and bovine gamma globulin (BGG) as the carriers. It has been stated in the literature that these proteins share common antigenic determinants and are thus immunologically cross reactive. This apparently has been due to the misinterpretation of experiments reported by Cohen & Hermes (A & R 5, 290). We have rigorously excluded antigenic similarities in these proteins.

Well documented, reduced immunoglobulin synthesis in humans with multiple myeloma has been shown not to be due to a decreased amino acid precursor pool. This suggested to us that it might be due to an inhibitor with activity similar to that demonstrated in antigenic competition. We have begun studies with mice bearing transplantable plasmacytomas and have demonstrated the presence of such an inhibitor. When given intravenously, it suppresses both the primary and secondary immune responses. It also inhibits mitogen induced lymphocyte proliferation. It is not cytotoxic, does not require the presence of complement, and is stable to freeze-thaw and 60°C for 1 hour.

In the course of the rabbit studies we have specifically purified high and low affinity antibody and determined equilibrium constants by fluorescence quenching. We are now involved with studies similar to those reported in last year's annual report designed to examine the influence of binding energy on efficiency of complement utilization during the interaction of antibody with cell bound antigen .

Significance to Biomedical Research and the Program of the Institute:

We believe that the nonspecific inhibitor of antibody synthesis plays an important role in the regulation of the immune response. It may be of great significance in those human diseases with associated acquired broad immune deficiencies.

Proposed Course:

Two manuscripts dealing with the rabbit work have been submitted for publication. The initial observations with the mice have been completed and we are preparing a manuscript. This work will be terminated at NIH due to departure of the principal investigator. The PI intendsto apply for extramural support for continuation of this work.

Honors and Awards: NONE

Publications:

1. Fauci, A. S., Frank, M. M., and Johnson, J. S.: The relationship between antibody affinity and the efficiency of complement fixation. J. Immun. 105: 215-220, 1970.

2. Fauci, A. S. and Johnson, J. S.: Antigenic competition at the cellular level: Response of rabbit lymph node cells to the trinitrophenyl on p-arsinilic haptens. J. Immun. In press.

3. Fauci, A. S., and Johnson, J. S.: Suppression of Antibody Synthesis I. Evidence for a Circulating Inhibitor of Antibody Synthesis Demonstrable at the Cellular Level. J. Immun. In press.

4. Fauci, A. S., and Johnson, J. S.: Suppression of Antibody Synthesis II. The Effect of Carrier-Specific Helper Cells upon Haptenic Competition. J. Immun. In press.

Serial No. NIAID-39(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Host Responses to Infection

Previous Serial Number: SAME

Principal Investigator: Dr. Richard K. Root

Other Investigators: Dr. Joseph S. Bujak
Dr. Harry R. Kimball
Dr. Michael M. Frank
Dr. Alan D. Schreiber*
Dr. Alan S. Rosenthal
Dr. Robert A. Clark
Dr. John N. Sheagren
Dr. Richard D. Diamond
Dr. John E. Bennett
Mr. Dominic J. Balestra

Cooperating Unit: Dr. Thomas P. Stossel, NHLI, NIH (Project no. NHLI-326)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2-11/12	1/12		3
Professional:	2-11/12	1/12		3
Other:	0	0		0

Project Description:

Selected patients studied on the ward and in the laboratory include:

a. Patients with recurrent bacterial infections including chronic granulomatous disease of childhood (CGD), Chediak-Higashi syndrome (CHS), cyclic neutropenia and neutropenia of diverse etiologies, hypogammaglobulinemia, and diabetes mellitus.

b. Patients with Wegener's granulomatosis

c. Patients with recurrent fevers

d. Patients with mucocutaneous moniliasis

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*Salaried by Medicine Branch, NCI, NIH

e. Patients with eosinophilia

f. Patients with systemic mycoses

Extensive studies are in progress concerning the nature of defects in host defenses responsible for recurrent infections in these patients, with particular attention paid to leukocyte functions.

Objectives:

Human Studies:

1. To examine the adequacy of host immunoglobulin levels, complement, delayed hypersensitivity and leukocyte responses and functions in patients with recurrent infections.

2. To define and attempt to correct the enzymatic deficiencies in leukocytes from patients with chronic granulomatous disease of childhood (CGD) responsible for their abnormal bactericidal and fungicidal activity.

3. To examine the phagocytic, bactericidal, fungicidal and metabolic functional capabilities of CHS leukocytes and compare these with CGD cells.

4. To examine phagocytic, bactericidal and metabolic properties of leukocytes from patients with neutropenia to see if cellular abnormalities exist that might be related to their neutropenia.

5. To examine serum and cell-mediated leukocyte functional abnormalities in patients with diabetes mellitus to seek to explain their increased propensity to infection.

6. To examine leukocyte functions in patients with Wegener's granulomatosis before and during therapy with cytoxan.

7. To examine leukocyte bactericidal, candicidal and metabolic activity in patients with mucocutaneous moniliasis.

8. To compare functional and metabolic properties of human monocytes and eosinophils vs. granulocytes.

9. To determine the mechanisms by which human macrophages kill bacteria.

10. To measure the effects of various metabolic inhibitors on phagocytosis and bactericidal activities of leukocytes.

Animal Studies:

1. To examine the role of C4 in the promotion of serum opsonic and bactericidal activity by studies on C4 deficient guinea pigs.

2. To determine the site within guinea pig granulocytes of the bactericidal reaction, iodination of protein.

3. To examine the relative uptake of RBC's coated with IgG, IgM and complement by macrophages.

Methods Employed:

In studies of host defense, serum is obtained for quantitative immunoglobulin levels (immunodiffusion), hemolytic complement and circulating antibodies. Leukocyte inflammatory responses are measured in vivo by peripheral counts, responses to infection and injections of endotoxin or etiocholanolone, and migration into skin windows. Chemotactic assays (Boyden chamber) and motility against gravity are used to measure in vitro leukocyte responses. Phagocytic function and serum opsonizing activity are tested by separating peripheral leukocytes from patient blood and mixing them with various particles, including polystyrene latex balls, Candida and bacteria in serum and buffer. Leukocyte bactericidal assays are performed by a pour plate technique. Metabolic functions are assessed by measurements of O_2 consumption (Gilson respirometer), hexose monophosphate shunt (C^{14}-1 glucose oxidation) and Krebs cycle (C^{14}-6 glucose oxidation) activity, hydrogen peroxide formation (C^{14}-formate oxidation) and reduction of nitro-blue tetrazolium dyes in resting and phagocytizing cells. Iodination of intracellular protein with phagocytosis is determined by incubating leukocytes with I^{125} and counting radioactivity in TCA precipitates of whole cells or subcellular fractions. Degranulation of lysosomes is followed by phase and electron microscopy and the appearance of enzymatic activity in phagocytic vesicles. The latter is determined by homogenizing leukocytes which have phagocytized mineral oil particles and placing the homogenates on a sucrose density gradient. After centrifugation, the mineral oil-laden phagosomes rise to the top of the gradient and can be assayed for enzymatic activity and iodinated protein.

Human monocytes are separated from whole blood by Ficoll-Hypaque gradient centrifugation, cultured on Petrie dishes or in flasks and their bactericidal and metabolic properties evaluated after 3 to 4 days of incubation and conversion to macrophages.

Serum is obtained from C4 deficient, normal and heterozygote NIH all-purpose guinea pigs and assessed for bactericidal activity against complement sensitive E. coli. Opsonic activity of the different sera is evaluated by measuring the uptake of radio-labelled bacteria by leukocytes in the presence of the different sera. This procedure permits frequent sampling and an examination of the kinetics of the ingestion process.

Cr^{51}-labelled erythrocytes are sensitized by exposure to γM or γG and complement and incubated with macrophages obtained from guinea pig spleens or peritoneal exudates. Uptake versus immune adherence is evaluated by microscopic exam and radioactive counting of leukocytes which have been incubated with erythrocytes.

Major Findings:

Chediak-Higashi Syndrome

1. Chediak-Higashi syndrome (CHS) granulocytes were found to have a broad defect in bactericidal activity which is most pronounced for gram-positive organisms. This defect is manifest primarily as a delay in killing, rather than the prolonged impairment characteristic of chronic granulomatous disease (CGD) leukocytes. Metabolic studies revealed no defects in peroxide production. Oxidation of C^{14}-1-glucose, and iodination of protein were found to be significantly greater than normal by non-phagocytizing CHS cells. Phase, electron microscopic and biochemical analysis of phagocytizing CHS indicated defective degranulation involving faulty delivery of acid hydrolase into CHS phagosomes. In addition, there is a failure of release of lysosomal enzymatic activity into cell-free supernatants by phagocytizing CHS leukocytes, which is characteristic of normal cells. These lysosomal functional abnormalities are believed to be responsible for the observed bactericidal defects.

2. CGD leukocytes were found to have normal levels of NADH oxidase activity, in contrast to some literature reports. Degranulation as measured by the appearance of enzymatic activity into CGD phagosomes was found to be normal. Treatment of CGD leukocytes with ascorbic acid promoted a tripling of the defective oxidation of C^{14}-1-glucose and slightly increased in vitro bactericidal activity against S. aureus. Ascorbic acid was also found to enhance phagocytic rates. Therapeutic attempts with ascorbic acid are underway.

3. Human monocytes in long-term culture were converted to macrophages, and evaluation of bactericidal activity against S. aureus revealed slower kill rates than that seen by monocytes and granulocytes. CGD macrophages lacked normal antistaphylococcal activity, suggesting that hydrogen peroxide production is necessary for normal macrophage bactericidal activity similar to that known for granulocytes.

4. A method which employs the radiolabelling of bacteria with C14 amino acids and measuring the uptake of these bacteria by leukocytes through scintillation counting of digests of the leukocyte pellet was developed to more precisely evaluate phagocytosis. This made it possible to study with ease and precision the rates of phagocytosis of different bacteria by different cells in the presence of different opsonic systems. Besides the time-saving feature, this technique has great advantages over microscopic methods since it permits the assessment of the phagocytic activity of the leukocyte population as a whole rather than the small sample routinely counted visually.

5. Comparative studies of the phagocytic and bactericidal activities of granulocytes and monocytes revealed that the former were capable of greater rates of phagocytosis and, therefore, more rapid bacterial killing. This is perhaps consistent with the major role played by these cells in the early response to inflammation.

6. Several compounds that have been reported to cause a dissociation between cellular phagocytic and respiratory activity were evaluated for their effects on phagocytosis by the sensitive radiolabelled bacterial uptake assay. It was found that all compounds (colchicine, n-ethylmaleimide and hydrocortisone in high doses) significantly suppressed phagocytosis and, in most cases, to a degree which paralleled the respiratory suppression. Since the previous studies did not make use of as sensitive a phagocytic assay, the interpretation of their findings as indicating a dissociation between phagocytosis and post-phagocytic metabolic events must be questioned.

7. The ability of sodium azide to inhibit the intracellular myeloperoxidase-mediated reaction of protein iodinization and bacterial killing reported by Klebanoff, *et al.*, in 1970 was confirmed.

8. Studies on the serum of C4 deficient guinea pigs disclosed diminished but not absent bactericidal activity against *E. coli* and opsonic activity for *S. aureus*, type 25 pneumococci and another strain of *E. coli*. These findings suggested the operation of a bypass mechanism in these animals, which directly activates C3 in the complement sequence. Treatment of C4D serum with heat (56° for 30 minutes), .01 M EDTA or zymosan abolished these activities and suggested that heat-labile factors, divalent cations, C3 and later components are operating in this bypass mechanism.

9. Human granulocytes and monocytes were found to be capable of the ingestion and inactivation of cryptococci. Peroxide-mediated mechanisms were shown to be important in intracellular killing of cryptococci. No abnormalities of opsonization, ingestion or destruction of these organisms by these cells were found in patients with cryptococcosis.

10. Studies concerned with the site of intracellular protein iodination in guinea pigs' granulocytes revealed that non-phagocytizing cells showed low levels of this activity in the lysosomal fraction. A five-fold increase in activity with a shift to the phagosomal fraction occurred in cells which had ingested mineral oil particles. Studies are underway to determine what protein is being iodinated.

Significance to Biomedical Research and the Program of the Institute:

These studies provide insight into the mechanisms (both humoral and cellular) which govern normal phagocytic and digestive activity of leukocytes and how these might be altered in various disease states. Such an understanding may conceivably lead to therapeutic correction of abnormalities and enhancement of normal activities when indicated.

Proposed Course: To be discontinued.

Honors and Awards: NONE

Publications:

1. Mickenberg, I. D., Root, R. K., and Wolff, S. M.: Leukocytic function in acquired hypogammaglobulinemia. J. Clin. Invest. 49: 1528-1538, 1970.

2. Root, R. K., Wolff, S. M., and Baer, H.: Skin reacitivity of human subjects to a polysaccharide component of BCG culture filtrates. Proc. Soc. Exp. Biol. Med. 136: 403-406, 1971.

3. Mickenberg, I. D., Snyderman, R., Root, R. K., Mergenhagen, S. E., and Wolff, S. M.: Immune fever in the rabbit: responses of the hematologic and complement systems. J. Immun. In press.

4. Mickenberg, I. D., Snyderman, R., Root, R. K., Mergenhagen, S. E., and Wolff, S. M.: The relationship of complement consumption to immune fever. J. Immun. In press.

Serial No. NIAID-40 (c)
1. LCI
2. Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1,1970 through June 30, 1971

Project Title: Clinical Studies on Patients with Known or Suspected Parasitic Diseases.

Previous Serial Number: SAME

Principal Investigators: Dr. Franklin A. Neva, LCI,NIAID
Dr. Peter T. Franck , LCI, NIAID
Dr. Theodore E. Nash, LCI, NIAID

Other Investigators: Dr. John N. Sheagren, LCI, NIAID
Mr. Deaner K. Lawless, LCI, NIAID

Cooperating Units: Dr. Myron Schultz, Parasitic Disease Drug Service, National Center for Disease Control
Dr. Donald Spigner, Peace Corps Medical Officer, Washington, D. C.
Dr. Martin Wolfe, State Department Medical Division

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2-3/12			2-3/12
Professional:	2-3/12			2-3/12
Other:	0			0

Project Description:

Objectives:

1. To establish or confirm specific etiologic diagnosis of parasitic infections by appropriate laboratory methods, and attempt to develop and evaluate new diagnostic methods.

2. To assess the quantitative relationship of parasitic infection to the patient's process.

3. To investigate the etiology of illnesses characterized by prominent eosinophilia, attempting to differentiate Parasitic eosinophilias from those of other origins.

4. In cases of schistosomiasis, to develop improved criteria for assessment of severity of disease, indications for treatment, and reversibility of the disease process.

5. To contribute to the overall training of clinical associates, guest workers, and outside physicians in clinical parasitic disease.

Methods Employed:

Clinical material continues to be actively recruited, in order to establish contacts in a variety of medical facilities which might contribute suitable patients. In cooperation with the Montgomery County Health Department, diagnostic and therapeutic services are offered to selected immigrants newly arrived from the tropics and parasitologic surveys conducted on such populations. Special diagnostic facilities such as serologic tests for amebiasis, toxoplasmosis, and filariasis are provided by research workers of the Laboratory of Parasitic Diseases. Consultation service for parasitic diseases are provided to the clinical center as well as to outside medical groups.

Major Findings:

This project remains in its early phases, with the addition of Drs. Franck and Nash, but somewhat hampered and delayed by the recent retirement of Mr. Lawless.

A variety of patients, two referred by the Peace Corps with urinary schistosomiasis, have been admitted for diagnostic evaluation and treatment. A series of patients with malaria have been studied in an attempt to correlate time of rupture of schisonts with fluctuations in platelet and white cell counts, complement levels, and onset of fever. A guest worker, Dr. David Hayman, cooperated with the Montgomery County Health Department in a survey to determine the prevalence of intestinal parasites among recently arrived Cuban immigrants. The results of this survey indicated a high prevalence of infection with a number of common helminths and protozoa, despite periods of residence in this area of six months or longer. Further studies on the host-parasite relationship of individuals from this group with persistent Giardia infection are planned.

Significance to Biomedical Research and the Program of the Institutes:

An active service and research unit serves to complement the various disciplines represented within the Laboratory of Clinical Investigation and helps provide a well-balanced training and research program in infectious diseases. In particular, application of some of the modern techniques of immunology to parasitology may prove of great benefit to both disciplines.

Proposed Course:

To continue as planned.

Honors and Awards: NONE

Publications:

1. Neva, F. A., Sheagren, J. N., Shulman, N. R., and Canfield, C. J.: Malaria: Host-defense mechanisms and complications. Ann. Intern. Med 73: 295-306, 1970.

Serial No. NIAID-41(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Resistance to Infection; the Role of Secretory and Systemic Immune Mechanisms

Previous Serial Number: NONE

Principal Investigator: Dr. Robert S. Northrup

Other Investigators: Dr. John S. Johnson

Cooperating Units: Dr. Alan Bernstein, Wyeth Labs., Marietta, Pa.
Dr. C. C. J. Carpenter, Johns Hopkins University, Baltimore, Md.
Dr. F. Chisari, DBS, NIH (Project Number DBS-44)
Dr. J. Feeley, DBS, NIH (Project Number DBS-22)
Dr. W. B. Greenough, Johns Hopkins University, Baltimore, Md.
Dr. Richard Hornick, University of Maryland, Baltimore, Md.
Dr. R. Kirschstein, DBS, NIH (Project Number DBS-44)
Dr. C. Miller, DBS, NIH (No Project Number)
Dr. Nathaniel Pierce, Johns Hopkins University, Baltimore, Md.
Dr. Marilyn Renfield, Children's Hospital, Washington, D. C.
Dr. Willard Verwey, University of Texas, Galveston, Texas

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	11/12			11/12
Professional:	11/12			11/12
Other:	0			0

Project Description:

Objectives:

To determine the role of secretory and systemic immune mechanisms in resistance to infection, and the response in these systems to infection and to immunization with bacterially-derived antigens, with an emphasis on infections confined to epithelial surfaces.

Methods Employed:

The aluminum hydroxide adsorption method of cholera toxin preparation was modified and standardized. Toxin and antitoxin titrations were set up. Cholera toxoid was used to immunize humans, and a local reaction was noted.

Intradermal injection of the toxoid was carried out in volunteers, and the reactions observed and examined histologically. Monkeys were immunized with this toxoid, and the resulting severe local reactions were biopsied and examined microscopically. Serologic responses of the monkeys and humans were analyzed, and lymphocyte stimulation in culture by the toxoid was examined. Other rhesus and patas monkeys were challenged intragastrically with V. cholerae organisms and/or cholera toxin, and clinical, bacteriologic, and serologic results were observed and analyzed. A pilot study comparing antibody responses in individual immunoglobulin classes in respiratory secretions and serum to pertussis, diphtheria and tetanus parenteral immunization was set up, utilizing an indirect fluorescent antibody technique for the pertussis antibody measurements. High titered dog serum shown to produce passive protection against oral cholera challenge was fractionated and specific antitoxin activity determined in immunoglobulin peaks. Similar methods were employed for serum from humans immunized with cholera toxoid. Methods for irreversibly inactivating cholera toxin, using combinations of formalin, heat, temperature, pH, and time, were investigated.

Major Findings:

A lightly formalinized cholera toxoid preparation (Wyeth lot 00101-DBS-IND-496) was found to produce local reactions which histologically resembled those of cholera toxin, although with a different time course. Reversion of the toxoid to toxin was subsequently concluded to be responsible. The human and monkey are the only species to demonstrate such a reaction to this toxoid after subcutaneous injection. A particularly marked humoral antibody response followed the severe reaction in monkeys. Lymphocyte studies and intradermal injections in volunteers suggested that cellular immunity did not play a major role in the local reaction to the toxoid.

The monkey was found to be minimally susceptible to clinical cholera after intragastric challenge with bacteria toxin plus sodium bicarbonate and milk. Histologic and serologic immune responses to the disease were observed.

Antitoxin activity was observed in both 19S and 7S immunoglobulin in immunized dogs, with specific 7S activity (antitoxin units per mg protein), only twice as high as 19S specific activity.

Significance to Biomedical Research and the Program of the Institute:

Pertussis and cholera are unusual diseases in that the causative bacteria do not penetrate the epithelial surface. These studies of the interrelationships between secretory and systemic immunity are thus particularly important in these diseases. In addition, the development of a cholera toxoid will allow stimulation of antitoxic immunity by immunization, which may produce more effective and longer lasting immunity against cholera infection or disease than present cholera vaccines. The observation of reactivity of the tested toxoid was instrumental in determining the lack of acceptability of the tested toxoid and has led to a search for other toxoiding techniques.

Proposed Course:

Further and more detailed analysis of the monkey response to cholera immunization or challenge will continue. Other human and dog sera will be fractionated and titered. More stable toxoids will be produced, after which studies of humans at optimum dosage levels can be carried out. The effect of chronic intrauterine antigenic stimulation by infection or Rh incompatibility will be studied in relationship to secretory and systemic immune responsiveness. Primary and secondary intestinal antigenic stimulation will be carried out to determine if secretory IgA is capable of demonstrating an anamnestic response.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-42(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synergy of Trimethoprim and Sulfamethoxazole in Cholera

Previous Serial Number: NONE

Principal Investigator: Dr. Robert S. Northrup

Other Investigators: NONE

Cooperating Units: Dr. Wiley H. Mosley, Cholera Research Laboratory
Dacca, East Pakistan (NIAID)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1/12			1/12
Professional:	1/12			1/12
Other:	0			0

Contract Support: NONE

Project Description:

Objectives:

To determine whether a combination of trimethoprim and sulfamethoxazole, shown previously to be effective in the treatment of cholera, acts synergistically or merely in an additive fashion.

Methods Employed:

Plate dilution studies have established sensitivity levels for the 2 drugs alone and in combination. Graded doses of the individual components will establish an effective dose of each, after which combination therapy will establish the minimal effective dose of the combination. Adult cholera patients being treated concurrently with fluid replacement will be used.

Major Findings:

The drugs act synergistically *in vitro* against classical *V. cholerae* strains and El Tor strains, although the synergy is less marked with the more sensitive El Tor *V. cholerae* strains.

Significance to Biomedical Research and the Program of the Institute:

The project, when completed, will represent the first objective demonstration of synergy of 2 antibiotics in patients. It will also allow determination of the dose of each component needed to produce the most complete clinical effect.

Proposed Course:

When patients and the political situation in Pakistan allow, the clinical extension of the completed *in vitro* studies will be carried out by Dr. Mosley and his staff.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-43(c)
1. LCI
2. Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Oral Administration of Stool Filtrates of Fetal Intestinal Organ Culture-derived Passage Material From Human Cases of "Nonbacterial Epidemic Gastroenteritis." Clinical Aspects.

Previous Serial Number: NONE

Principal Investigator: Dr. Robert S. Northrup

Other Investigators: Dr. David Fedson

Cooperating Units: Dr. Neil Blacklow, LID, NIAID, NIH (No Project Number)
Dr. R. Dolin, LID, NIAID, NIH (No Project Number)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	3/12			3/12
Professional:	3/12			3/12
Other:	0			0

Project Description:

Objectives:

To perform detailed clinical observation on the diarrheal syndrome induced in Clinical Center volunteers by a bacteria-free stool filtrate from patients with "winter vomiting" syndrome.

Methods Employed:

Careful balance records and electrolyte measurements on stool and seru determine the extent and significance of diarrhea and vomiting. Jejunal aspiration for bacteriological and immunological examination and intestinal absorption studies and small intestinal biopsy determine changes in these various parameters in the small intestine produced by the syndrome.

Major Findings:

Coliform invasion of the upper intestine was not associated with the syndrome. Xylose absorption appeared to be altered acutely. The titer of the challenge pool was at least 100 infectious doses per mg and less than 10,000. Volunteers appeared to be resistant to repeat challenge.

Significance to Biomedical Research and the Program of the Institute:

Better understanding of the pathophysiology of this very common epidemic disease will lead to methods for its prevention and effective treatment.

Proposed Course:

Following completion of the clinical studies described above, investigation will shift toward exploration of immune responses to the inducing gent, and development of preventive techniques.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-44(c)
1. LCI
2. Biochemistry and Immunochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Pathophysiology of Autoimmune Hemolytic Anemia

Previous Serial Number: NONE

Principal Investigator: Dr. Michael Frank

Other Investigators: Dr. Alan Schreiber*
Dr. Richard Root

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1-4/12	11/12		2-3/12
Professional:	10/12	11/12*		1-9/12
Other:	6/12	0		6/12

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with acute hemolytic anemia and to determine the effect of these substances on red cell survival. To this end a model of immune clearance of erythrocytes has been established in the guinea pig.

Methods Employed:

Rabbits have been immunized with guinea pig erythrocytes utilizing a number of different routes of injection, and IgM and IgG immunoglobulin fractions of high purity have been prepared from the rabbit antisera. Fractions containing antibody with high avidity for guinea pig erythrocytes have been used to sensitize guinea pig erythrocytes. The number of complement fixing sites per erythrocyte formed by these antibodies has been established by use of the C1a fixation and transfer test. The antibodies have been used to sensitize guinea pig erythrocytes labeled with chromium 51 and the half life of the cells studied after reinjection into guinea pigs. In some experiments the immunoglobulin fractions have been radiolabeled with ^{125}I by the Iodine Monochloride method and the absolute number of molecules of antibody on the cell surfaces determined by radioactive I uptake.

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*Salaried by Medicine Branch, NCI, NIH

Major Findings:

Our observations indicate that the half-life of chromated erythrocytes in the guinea pig is seven and one-half days. We find that sixty complement fixing IgM sites per cell are required before the cells show increased rates of clearance. We have independently shown that in this system, 1 IgM molecule can fix 1 C1 molecule, and 60 complement fixing units represent sixty IgM molecules per cell. When cells with sixty or more IgM sites are injected into the guinea pig, they are immediately cleared by the liver and then re-released into the circulation where they undergo a normal survival. The situation with respect to IgG sensitized cells is entirely different. As few as 1.4 complement fixing sites per erythrocyte are sufficient to lead to decreased survival of erythrocytes. To achieve 1.4 complement fixing sites per erythrocyte, about 2,000 IgG molecules per cell required as shown by the radioactive uptake of IgG antibody. These cells are not sequestered in the liver within five minutes of injection but undergo a straight-line, decreased survival curve for many hours with sequestration in the spleen. They are not re-released into the circulation. A small proportion of the IgG-coated cells do show normal subsequent survival after a period of several hours. That complement is important in the clearance of these cells has been shown by parallel studies in our newly described strain of guinea pigs with the total, genetically controlled deficiency of C4. In these animals IgM sensitization produces no sequestration of the cells and the cells undergo a normal survival. In the case of IgG, there is slightly decreased survival of cells with a very large number of IgG molecules per cell in C4 deficient animals; however, this does not approximate the rate of clearance noted in animals with intact complement function.

Significance to Biomedical Research and the Program of the Institute:

These studies are significant for two reasons. First, they go far in illuminating the role of antibody and complement in the clearance of cells in patients with the disease, autoimmune hemolytic anemia. They represent the first molecular studies of the effect of immunoglobulin and complement on the survival of erythrocytes. They explain the finding, known for many years, that patients with hemolytic anemia may have Coombs positive cells in their circulation, which have a perfectly normal survival. Presumably, the cells have complement components placed on their surface by IgM. Secondly, these studies point out the in vivo importance of earlier studies from this laboratory, demonstrating that IgM and IgG handle complement components differently and produce different kinds and amounts of damage to cell surfaces. This may have much wider medical significance beyond their importance in autoimmune hemolytic anemia. For example, tissue destruction and cell death is thought to be mediated by antibody and complement in many different diseases. We feel that the red cell model may be a relatively simple and accurate model of these situations *in vivo*.

Proposed Course:

This work will be completed. Further studies will be done to attempt to define the nature of the site created on red cells by antibody and complement which is quantitatively different for IgM and IgG antibodies. An attempt will be made to broaden these studies to include clearance in human beings in the future.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-45(c)
1. LCI
2. Electron Microscope Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural Correlates of the Cellular Events in Host Defense Mechanisms in Man and Experimental Animals

Previous Serial Number: NONE

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators:

Dr. David L. Rosenstreich	Dr. David Dale
Mr. J. Thomas Blake	Dr. Burton West
Dr. Sheldon Wolff	Dr. Richard Root
Dr. Harry Kimball	Dr. Michael Frank
Dr. Charles Kirkpatrick	Dr. Anthony Fauci
Dr. Robert Rich	Dr. Ethan Shevach

Cooperating Units:
Dr. John Finnerty, LPD, NIAID, NIH, Project No. NIAID 117-(d)
Dr. Ira Green, LI, NIAID, NIH, Project No. 143
Dr. John Martin, LI, NIAID, NIH, No Project No.
Dr. Robert Scott, LEP, NIAID, NIH, No Project No.
Dr. John Van Boxal, LI, NIAID, NIH, Project No. 143

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2-7/12			2-7/12
Professional:	2-7/12			2-7/12
Other:	0			0

Project Description:

Objectives:

1. Provide ultrastructural analysis of the cellular events in clinical disorders of the host differences in man and experimental animals.

2. Develop new morphologic techniques for investigating animal models of human disease.

3. Provide support for other investigators in their clinical and experimental approaches to human disease.

Methods Employed:

Methodologic approaches are as varied as the disorders to which they have been applied but in general involve fixation of tissue specimens or material provided by the collaborating units. These specimens are subjected to autoradiographic, cytochemical, ultrastructural and morphometric analysis by standard techniques. Where specific methodologies have been specifically developed, they will be briefly described under subject headings reviewed under Major Findings.

Major Findings:

1. Drs. David Dale, Robert Scott and Sheldon Wolff - Ultrastructural analysis of bone marrow and peripheral blood of cyclic neutropenia in gray collies.

We have studied peripheral blood and marrow of normal and cyclic neutropenic dogs and have observed that cycles of neutropenia correlated with striking marrow metabolic activity and release of neutrophiles with increased glycogen deposits within cytoplasmic sites. No similar lesions were present in other cell series or in normal dogs.

2. Dr. Burton West and Dr. Harry Kimball. Isolation of Human Neutrophil granules by Sucrose Isopyicnic Gradients.

A biochemical analysis of gradient separated granules has been subjected to a corresponding electron microscopic analysis. Micrographs of granule fractions correlated morphologically with segregation of enzymatic activity into distinct classes.

3. Drs. Robert Rich and Charles Kirkpatrick. Photosensitive Chelitis.

We have described a case of u.v. light induced chelitis associated with ultrastructural evidence of nuclear bodies and cytoplasmic smooth endoplasmic reticulum particles resembling the myxovirus like particle seen in systemic lupus erythematous glomeruli by electron microscopy. These lesions were reversed by shielding from u. v. lights.

4. Dr. Richard Root. Ultrastructural Correlate of impaired granulocyte bactericidal activity in Chediak-Higashi Disease (CHD).

We have characterized by electron micrscopy the impaired killing of bacteria by CHD granules and shown that the abnormal CHD granules exhibit delayed fusion with phagosomes. By use of peroxidase cytochemical techniques we can show that myeloperoxidase, an enzyme necessary for bactericidal activity, through an halogenation mechanism is retained within the abnormal granules and is not released in a normal fashion.

5. Drs. Michael Frank, Ira Green and John Van Boxel. Does cytotoxic killing involve complement?

Red blood membranes were prepared and examined for complement specific lesion or "holes". Studies as yet incomplete have failed to identify such lesions but immunologic data do suggest a specific involvement of γ_2 immunoglobulin.

6. Drs. John Finnerty and John Martin. A technique for the isolation of infectious plasmodia bergeii free of host red blood cell membranes.

P. Bergeii infected mouse red blood cells were exposed to Tris-NH_4CL lysis and the biologic properties of the isolated P. Bergeii studies. Electron micographic analysis demonstrated that the infectious organisms are undamaged and free of host membrane. Immunization with such preparation yields cytotoxic P. Bergeii specific antibodies. This represents the first such isolation procedures free of containing host material.

7. Drs. Green and Shevach. Ultrastructural membrane receptors on guinea pig L_2C leukemic cells.

The surface membranes ultrastructure of guinea pig L_2C leukemia cells have been examined. Preparation of peroxidase and/or ferritin markers to immunoglobulin surface determinents is being applied to an analysis and mapping of their distribution.

8. Drs. Anthony Fauci and Sheldon Wolff. The ultrastructure of the kidney in Wegner's Granulomatosis.

Sections of kidney from patients with Wegner's granulomatosis have been fixed with gluderaldehyde-osmic acid and embedded in plastic. Thin sections will be examined by light and electron microscopy both before and after successful therapy with immunosuppressive drugs. Preliminary observations indicate adequate fixation and preservation of structure.

Significance to Biomedical Research and the Program of the Institute:

The significance of these studies can be best appreciated by the clear need for a new approach to the diseases being studied. It is hoped that an analysis of the ultrastructural correlates of these diseases will provide new insight into an understanding of the disease mechanisms involved and provide an avenue for these needed new approaches both for therapy and prevention.

Proposed Course:

1. Complete studies in progress.

2. Expand areas of promise commenserate with the available material.

3. Suggest from electron microscopic data new biologic approaches to problems already under study.

Honors and Awards: NONE

Publications:

1. Rosenthal, A. S., Moses, H. L., and Ganote, C. E.: Interpretation of phosphatase cytochemical data. J. Histochem. Cytochem. 18: 915-916, 1970.

Serial No. NIAID-46(c)
1. LCI
2. Electron Microscope Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunobiologic and Ultrastructural Characterization of Cellular Immune Effector Cells.

Previous Serial Number: NONE

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. David L. Rosenstreich
Mr. J. Thomas Blake

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	11/12			11/12
Professional:	11/12			11/12
Other:	0			0

Project Description:

Objectives:

1) To develop and apply techniques for the isolation of cellular immune effector cells.

2) To characterize in a quantitative and qualitative fashion the response of such cells to a variety of *in vitro* correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and macrophage inhibitory factor production.

3) To characterize the ultrastructures of cellular immune effector cells as contrasted with other types of lymphocytes.

4) To study the mechanisms of lymphocyte activation by antigen and mitogen using visible antigenic markers such as horseradish peroxidase (HRPO) and HRPO-protein conjugates.

5) To study the regulation of cellular events and the role of cell cooperation and its relationships to humoral immunity.

6) Characterize the role of macrophages in cell activation.

Methods Employed:

1) Determination of DNA synthesis of lymphocytic populations with and without addition of antigen by 4 hour pulse of ^{3}H-TdR incorporation. Results assayed by either/or both autoradiography or liquid scintillation spectrometry.

2) Preparation and isolation of partially purified population of lymphocytes from oil induced peritoneal exudates or regional draining nodes subsequent (2-4 weeks) to immunization of inbred strain 2 or 13 guinea pigs with chemically defined antigens such as horse spleen ferritin, horseradish peroxidase or hapten protein conjugates.

3) Column purification: Peritoneal exudate cells (PEC) or lymph node cell suspensions were passed over glass bead columns and incubated at 37°C for 1 hour. Cells were then eluted with 10% guinea pig sera in minimal essential Eagle's media and the recovered cells studied.

4) Ultrastructural analysis of antigen localization by means of autoradiography (isotopically labelled antigen) or cytochemistry (peroxidase coupled or direct use).

Major Findings:

1) Complex lymphoid cell mixture placed over glass bead columns are depleted of antibody forming cells and their precursors as measured by loss of plaque forming cells (PFC'S); immunoglobulin and antigen specific receptor bearing cells as measured autoradiographically. By contrast those cells which participate in *in vitro* correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and elaboration of macrophage inhibitory factor (MIF) are excluded from the column.

2) There appears to be within the lymphoreticular system a segregation both functionally and anatomically of immune competence. Those cells which mediate cellular immune function appear to be sequentered within inflammatory sites such as oil-induced peritoneal exudates. These sequestered cells possess enhanced immune responsiveness producing greater inhibition of macrophage migration by a factor of 10 than an equivalent number of lymphocytes derived from the region nodes. Moreover, antigen recognition as indicated by new DNA synthesis in inflammatory exudate lymphocytes is 30-200 fold greater than an equivalent number of lymph node lymphocytes. These differences can not be explained by presence or absence of macrophages, significant blastogenic factor release or desparities in the kinetics of cell responsiveness.

3) The peritoneal exudate lymphocyte have unique surfaces membrane changes on ultrastructural analysis.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of an enriched population of cellular immune effector cells should provide a useful system for study of the role of the cellular immune effector cells in immunologic surveillance and their contribution to the humoral limb of host defenses. Tumor immunity and transplantation immunity have been shown to rely to a great extent upon mechanisms of cellular immune function such as cytotoxicity and elaboration of other so-called "soluble mediators." A more fundamental understanding of the immunobiology of these crucial events and morphologic correlates may provide new insights into immune competance in man and information on the mechanisms of cellular regulation of the immune responsiveness. Potential approaches to therapeutic manipulation of such events would be of immediate clinical relevance.

Proposed Course:

1. Characterize the regulation of lymphocyte activation.

2. Characterize the ultrastructures of cellular immune effectors.

3. Characterize the localization and fate of antigen in *in vitro* antigen-mediated lymphocyte proliferation.

4. Explore the role of cellular immune effector in cell - cell interaction and cooperative activity with those cells required for antibody formation and transplantation immunity.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-47(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Influence of Fever on Drug Metabolism

Previous Serial Number: NONE

Principal Investigator: Dr. Chull S. Song

Other Investigators: Dr. Sheldon M. Wolff, LCI, NIAID
Mrs. Nancy A. Gelb, LCI, NIAID

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1-5/12			1-5/12
Professional:	11/12			11/12
Other:	6/12			6/12

Project Description:

Objectives:

1) To study the influence of artificially induced fever on the following three parameters of drug metabolism in man: glucuronidation, sulfation, and hydroxylation.

2) To study the influence of artificially induced fever on overall pharmacokinetics of drug disposal in man.

Methods Employed:

1) Salicylamide was chosen as a model drug because of its metabolism in man via the three pathways listed above, its short half-life, its proven safety, and the availability of reproducible methods for assaying its metabolites. Its major pathway of metabolism, furthermore, is via glucuronidation, and the data in literature strongly indicated that the mechanism of its glucuronidation was closely related to that by which hepatic glucuronidation of bilirubin takes place.

2) Normal human volunteers were given a steroidal pyrogen (etiocholanolone, 0.3 mg/kg im) or a bacterial pyrogen (Lipexal, 5 mμg/kg iv) to induce fever.

3) Single oral doses of salicylamide, 1 g in 500-600 ml of H_2O, were given to the volunteers at the height of fever, and urinary excretion of all metabolites were determined at 30-minute intervals for 4 hours. In 5 volunteers, simultaneous plasma levels of free as well as glucuronidated salicylamide were determined.

4) All studies were then repeated one week later, without administration of pyrogens, to establish control values. Several volunteers underwent two control studies, one week apart, to verify the fact that pre-treatment with salicylamide does not alter its subsequent metabolism.

Major Findings:

1) During the four-hour period of study, over 90% of the administered drug is recovered in the urine as metabolites in all studies.

2) Fever produced by etiocholanolone or endotoxin consistently caused decreases in the rate of glucuronidation of salicylamide. The average proportion of salicylamide excreted as glucuronide decreased from 61% during control to 55% during febrile periods.

3) The rates of sulfation and hydroxylation were increased during fever, so that there was no significant difference in the overall recovery of the drug during the four-hour experimental periods.

4) Fever significantly shortens the apparent half-life of excretion of salicylamide as determined by the initial rates of excretion of drug metabolites.

Significance to Biomedical Research and the Program of the Institute:

We have found that artificial fever has a substantial influence on the metabolism of a model drug, salicylamide. Of clinical significance is the depression of glucuronidation of salicylamide by fever. This finding may be relevant to situations in which the hepatic glucuronidation is limited, e.g. patients with Gilbert's syndrome in whom we found (in a collaborative study with Drs. Berk and Blaschke of NCI) about 52% glucuronidation of salicylamide administered under identical (afebrile) situations. Further depression of glucuronidation during spontaneous febrile illnesses in these patients, when coupled with other abnormalities in bilirubin transport, may cause the frequently noted increases in jaundice and unconjugated hyperbilirubinemia. Certain drugs of frequent use in association with febrile diseases, e.g. chloramphenicol, are metabolized primarily by means of hepatic glucuronidation. Thus, a prolonged fever in man may significantly delay the metabolism of such potentially toxic drugs.

Proposed Course:

To be discontinued.

Honors and Awards: NONE

Publications: NONE

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LABORATORY OF INFECTIOUS DISEASES

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Summary Statement
Annual Report of the Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
July 1, 1970 through June 30, 1971

As in past years the major interest in the Laboratory of Infectious Diseases continues to be the natural history of acute infections of the respiratory and alimentary tracts. Considerable thought and effort have also been directed toward the development of methods for the control or prevention of such infections. The rate of progress has been gratifying in most of these areas, and there is increasing cause for optimism that current efforts at genetic manipulation of the important respiratory tract pathogens will lead to safe,effective live vaccines for prevention of disease.

Hepatitis. During the past year the central focus of hepatitis research has remained the Australia antigen (or HAA). Although attempts to propagate the agent *in vitro* met with failure, we were able to extend our understanding of the epidemiology of HAA infection through serologic and sero-epidemiologic studies which were made possible by the development of a highly sensitive, specific radio-immunoprecipitation (RIP) assay for antibody to HAA (anti-HAA). A double-antibody method (using anti-human globulin serum to precipitate antigen-antibody complexes) was employed, and the technique was adapted to the microtiter system. With the RIP technique it was possible in many instances to detect anti-HAA following infection with HAA, whereas the CF assay uniformly yielded negative results. In collaborative studies performed with Dr. Krugman we examined the anti-HAA response of children experimentally infected with HAA (the MS-2 agent). An antibody response to HAA was detected in the 3 children tested who had transient HAA antigenemia and in one of 3 children who became chronic carriers of the antigen after MS-2 hepatitis. In contrast, none of the 8 children who developed the short incubation or MS-1 type hepatitis had such an antibody response. Similarly adults who developed HAA-positive hepatitis following artificial or natural exposure to icterogenic materials often developed RIP antibody for HAA. By using antibody directed against individual globulin classes in the second part of the RIP test, we were able to measure the relative contribution of IgM and IgG antibodies to the anti-HAA response. Various patterns of IgM and IgG response were observed: in most adults both IgM and IgG antibodies to HAA could be detected, whereas in children little IgM antibody was found.

Evidence obtained from long-term follow-up of persons with HAA-positive hepatitis suggested that antibody detected by RIP persisted for years following hepatitis. To determine the age specific frequency of anti-HAA we tested sera from over 300 persons of all ages who resided in the Washington, D. C., metropolitan area. Antibody was detected infrequently in sera from infants and children, whereas a significant proportion of young adults was found to be positive, and the frequency of antibody reached a peak of 39% in middle aged adults. Thereafter there was a slight diminution in frequency of antibody in the elderly. These findings suggest that HAA-positive hepatitis is primarily a disease of adults, a finding in good agreement with results obtained by others who found HAA more frequently in adults than in children. The results of the seroepidemiologic survey further suggest that HAA infection is often subclinical and is frequently spread by means other than parenteral inoculation, most likely the oral route (Purcell, Lander).

Acute non-bacterial gastroenteritis. One of the notable successes of the past year was achieved in one of the newest programs in LID. We tested filtrates of fecal material which were derived from 4 separate outbreaks of acute non-bacterial gastro-enteritis which were suspected of being viral in origin based upon the pattern of the outbreak and failure to recover a bacterial pathogen. An anal swab filtrate from only one of these outbreaks induced an acute, self limited disease in adult volunteers. Bacteria-free material from the Norwalk, Ohio, outbreak induced a spectrum of effects in volunteers ranging from a predominantly diarrheal disease without vomiting to an illness in which only vomiting occurred. The Norwalk agent was transmitted through 3 passages in volunteers, and when one considers the resulting dilution of the original anal swab material, it is highly improbable that a non-replicating material, such as a toxin, was responsible for the disease manifestations seen in our volunteers. The Norwalk agent, which has remained consistently free of pathogenic bacteria, was analyzed by the rabbit ileal loop assay, and there was no evidence that bacterial enterotoxins were present.

All attempts to cultivate the organism in standard tissue cultures or human fetal intestinal organ culture failed. The organism may have grown in these systems, but using a variety of techniques we were not able to detect its presence. Nevertheless the following properties were defined by the appropriate studies in volunteers. The disease-producing potential of the Norwalk agent passed through 110 and 60 nm gradacol filters and resisted ether and an acid pH of 2.5. The first passage stool suspension produced disease at a 10^{-2} dilution, but not when diluted 10^{-4}. Nasopharyngeal washings from an ill volunteer did not appear to contain the agent. There was evidence for homologous immunity lasting for at least 2 months. In summary the agent is probably a small, non-lipid containing, acid stable virus which confers at least short term immunity.

We plan to press on as rapidly as possible in this area with special emphasis on development of an *in vitro* assay system for the Norwalk agent. Specimens from other outbreaks will be studied, and the relationship of any new agent to the Norwalk agent will be determined. The successes of the past year are particularly exciting because the area of epidemic non-bacterial gastroenteritis is largely uncharted despite the public health importance of this type of disease (Dolin, Blacklow, Chanock; Fedson and Kasel, LCI, and Dupont and Hornick, U. of Md.).

Mycoplasma. At present, metabolic inhibition (MI) and complement fixation (CF) are the most widely used methods for serodiagnosis of *M. pneumoniae* infections, but methods with increased sensitivity for detection of antibody are needed to illuminate certain unexplained aspects of *M. pneumoniae* infection. For example, there is the unusual age distribution of disease which occurs with greatest incidence in the 5-15 year age group. Then, there is the recent experience at Children's Hospital of D. C. in which an unexpectedly high frequency of shedding of *M. pneumoniae* was observed in young children who were not ill. Taken together these findings suggest that *M. pneumoniae* disease might result from re-infection of individuals previously sensitized to the organism. In order to explore this possibility highly sensitive methods for assay of *M. pneumoniae* antibody are required.

To meet the needs just described two highly sensitive serologic tests for *M. pneumoniae* antibody were developed. Using C^{14} oleic acid and C^{14} palmitic acid labelled organisms a radioimmunoprecipitation (RIP) technique was devised. In addition the complement mediated killing reaction first described by Gale and Kenny was made more sensitive by altering the conditions of the test. Both the RIP and mycoplasmacidal techniques were found to be considerably more sensitive than the MI method.

In a preliminary study, infants 7 to 12 months of age, without respiratory tract disease, were found to have a significantly lower mean mycoplasmacidal serum antibody titer and frequency of antibody than children one to two years of age who were also free of respiratory disease. Similarly the infants had a significantly lower titer than a group of normal adults. The high frequency and titer of antibody in sera of one to two year old children gives some preliminary support to the hypothesis that disease might be favored by prior sensitization induced by infection at an early age.

Killing of *M. pneumoniae* by antiserum was shown to be mediated by antibody specific for the glycolipid hapten of the organism. In the presence of complement the organism was killed by rabbit serum which contained antibody for the glycolipid hapten but not for the other constituents of *M. pneumoniae*. Apparently, viability was destroyed by lysis of the mycoplasmal membrane effected by antibody and complement operating in concert. When viewed under the electron microscope, the affected organisms were seen to change from filamentous structures to larger circular structures with many tears or holes in the limiting membrane. Additional evidence that the membrane had been compromised was provided by the observation that complement-mediating killing was associated with release of 260 nm absorbing material, presumably nucleic acid.

The benign nature of infection produced by ts mutants of *M. pneumoniae* in the hamster was confirmed. Initially we did not detect revertants of the ts mutants among isolates recovered from infected hamsters. More intensive search for such revertants, however, revealed their occurrence in hamsters infected with several of the ts mutants which appeared to be most promising for use in a live *M. pneumoniae* vaccine. It was of interest that the hamsters which yielded revertants had a benign infection characterized by minimal pulmonary pathology. Although the ts mutants produced a limited attenuated infection, it was sufficient to induce significant resistance to the pathologic effects of virulent *M. pneumoniae* organisms. Current efforts are aimed at producing a ts mutant (or mutants) with a reversion rate that is so low that emergence of wild type organisms during infection will not occur with any appreciable frequency (Brunner, Chanock).

Ecology of respiratory tract pathogens in infants and children. *RS virus*. The available evidence suggests that immunologic factors play an important role in the pathogenesis of serious RS virus disease of infancy. It is likely that other immunologic mechanisms act to protect the host with previous RS virus experience from serious disease during subsequent re-infection. Studies performed last year suggest that local respiratory tract antibody constitutes one of these protective immunologic forces. Efforts to study the local antibody response in young infants were hampered by the presence of what appeared to be a non-specific inhibitor in nasal secretions. This inhibitor was not related to previous experience with RS virus, and for this reason was considered to be non-specific. Characterization of the inhibitor indicated it sedimented more rapidly than 11S IgA and was not IgM. Although the inhibitor tended to obscure a specific local antibody response, it was possible to detect such a response in a significant proportion of infected children /Wright, Chanock and Parrott (Children's Hospital, D. C.)/

Genetic studies with influenza and RS viruses. It is now clear that temperature-sensitive (ts) mutation leads to attenuation of influenza virus as indicated by restriction of virus growth in the lungs of experimental animals. Attenuation appears to be a phenotypic manifestation of the ts lesion(s) since transfer of the lesion(s) by recombination leads to an associated transfer of attenuation to the recombinant virus. This also means that live vaccines can be updated at the time new antigenic variants emerge by recombination and transfer of the ts lesion(s) to the new virus. The success of this approach was demonstrated this year when we transferred ts lesions (and the associated attenuation) of an influenza A2/1965 virus to the influenza A/Hong Kong/1968 virus. Of course this method of transferring attenuation to new influenza viruses requires that the ts lesions not affect that portion of the genome which codes for the hemagglutinin. Since we were fortunate in inducing ts mutations in the non-hemagglutinin portion of the genome of the 1965 influenza A virus, the transfer of ts lesions and attenuation to a new influenza virus could be achieved.

In addition to demonstrating the feasibility of the ts approach to attenuation of influenza virus several interesting observations concerning the nature of the mutations were made. In tests with the original 1965 ts mutants and those performed with the Hong Kong 1968 ts recombinants there was a strong association between degree of temperature defectiveness *in vitro* and the extent of limitation of growth *in vivo*. This relationship, which was not surprising, should prove useful for the purpose of predicting level of attenuation of new mutants or recombinants in future studies. Finally, we were able to identify 3 discrete ts lesions in the 2 mutants which were studied most extensively. One of the mutants, ts-1, contained 2 separate lesions--one lesion imposed a temperature limitation of 37°C, while the other produced a limitation of growth at 39°C. The second mutant, ts-2, had a distinct lesion which also imposed a limitation of growth at 39°C. This information should prove useful in the future in identifying similar or distinct lesions present in other mutants or recombinants. In this context it should be mentioned that the existence of 2 distinct lesions (or clusters of lesions) in the ts-1 mutant did not become evident until recombination with the Hong Kong virus yielded 2 classes of recombinants which complemented each other (Murphy, Nusinoff, Chanock).

Recognition of 3 complementation groups among the 7 ts mutants of RS virus was accomplished during the past year. This means that defects in at least 3 distinct cistrons have been identified. Members of a complementation group share a defect in at least one cistron, and this cistron is not affected in mutants belonging to the other 2 complementation groups. This information is of importance to the program of vaccine development in that we can now identify mutants which share a common defect. This simplifies the choice of mutants to be evaluated in man since in initial trials a representative from each complementation group should be tested rather than a series of mutants with the same defect (Wright).

Evaluation of a temperature-sensitive mutant of RS virus as a potential vaccine strain. Special emphasis has been given to the development of a live attenuated strain of RS virus for use in infants because of the increasing evidence that local respiratory tract defenses play a major role in resistance to infection. The ts-1 mutant of RS virus, induced by the chemical mutagen 5-fluorouridine, was chosen for the initial trials on the basis of its behavior *in vitro* in tissue culture and *in vivo* in the experimental hamster host and in adult volunteers. In adult volunteers infection was silent, and there was no evidence that the mutant reverted to wild type (Wright and Chanock).

To date 14 children have been given the ts-1 mutant, which has an *in vitro* shut-off temperature of 37°C. Six children were 4-6 years old, 6 were 2-4 years old, and 4 were 1-2 years old. Thirteen of the 14 children became infected, but respiratory illness did not occur. Thus far 20 isolates from 6 of the children who shed virus have been analyzed for evidence of reversion. All but 2 of the isolates retained the ts property. Two wild type viruses recovered from an 18 month old child produced plaques with high efficiency at 39°C, a temperature which was completely restrictive for the ts-1 mutant. Although the child was kept in relative isolation in the Clinical Research Center at Children's Hospital of District of Columbia, one cannot rule out the possibility of hospital-acquired super-infection with naturally occurring wild type virus. Since this is unlikely, we must tentatively conclude that reversion occurs in a small minority of infections. The pattern of shedding exhibited by the child who yielded wild type virus suggests that revertant virus did not take over and rapidly replace the ts mutant; revertant virus was detected on day 7, but only ts virus was recovered the next 2 days. In any case the sporadic shedding of apparent revertant virus was not associated with respiratory tract disease /Parrott (Children's Hospital, D. C.), Wright and Chanock/.

Evaluation of temperature-sensitive (ts) mutants of influenza virus in volunteers. Efforts to evaluate attenuation of the ts mutants of influenza A2/1965 were frustrated by our failure to find volunteers who lacked serum antibody for this virus. This was not surprising since the A2 viruses have been active for the past 13 years. The ts-1 and ts-2 mutants infected 50% of men who were selected for the lowest serum neutralizing antibody titer (1:32 - 1:1024), but illness did not occur. However, this does not mean that the mutants were attenuated since the wild type strain, similarly, did not produce disease. When it became clear that the ts mutants of influenza A2/1965 could not be assessed for loss of virulence, another approach was taken. Since 10-20% of our volunteer population lacked detectable serum neutralizing antibody for the Hong Kong influenza A virus, we produced recombinants with the hemagglutinin of Hong Kong and the ts defect(s) of influenza A2/1965. A Hong Kong x ts-1 recombinant (with a shut-off temperature of 37°C) failed to infect any of 13 seronegative men when an inoculum of 10^5 pfu was given. In contrast a Hong Kong x ts-2 recombinant (with a shut-off temperature of 39°C) infected each of 9 seronegative volunteers and produced a mild common cold-like illness. Each of the virus isolates retained the ts property indicating that the recombinant was stable in vivo. When these men were challenged subsequently with wild type virus, they were resistant.

Although a final influenza vaccine product did not emerge from this study, we have advanced a considerable distance toward this goal. It is clear that ts mutations in influenza virus result in attenuation of virulence for man. The degree of attenuation is related to the degree of temperature sensitivity. The ts-2 recombinant with a shut-off temperature of 39°C was moderately attenuated, while the ts-1 recombinant was over-attenuated in that it did not infect when given at a dosage of 10^5 pfu. Also, we now know that it is possible to attenuate influenza virus by introducing a ts defect into the virus through the mechanism of recombination. Furthermore, infection with an attenuated ts mutant (the Hong Kong ts-2 recombinant) induced significant resistance to challenge with wild type virus. Finally, the ts-2 recombinant was stable in vivo since we did not detect reversion to wild phenotype. What is probably required for immunization against contemporary influenza A viruses is a ts mutant which is intermediate in defectiveness between the ts-1 and ts-2 recombinants, possibly a ts mutant which has a shut-off temperature of 38°C. Once we have identified the appropriate in vitro laboratory marker, i.e., temperature limitation, it should be possible to update live influenza virus vaccines to meet the threat of new antigenic variants by transferring ts lesions to the new virus through recombination (Murphy, Chalhub, Chanock).

Rhinoviruses. A chemically induced ts mutant of rhinovirus 1A was administered by the intranasal route to seronegative volunteers. Almost all of the men became infected and the majority developed typical rhinovirus illness. This mutant which had an in vitro shut-off temperature of 38°C was apparently not sufficiently defective to have the desired degree of attenuation. Analysis of the virus isolates from the volunteers indicated that the emergence of wild type revertants was not responsible for the occurrence of disease. Recently a ts mutant of rhinovirus 1A with a shut-off temperature of 37°C was produced, and it is planned that this mutant will be tested in volunteers during the coming year (Perkins, Buscho).

Intranasal vaccination with inactivated rhinovirus 13 induced resistance to homologous live virus challenge which lasted for at least 6 months. The local antibody which developed in the nasal secretions was specific for type 13 virus, and this antibody persisted for at least 14 months. Finally we were able to obtain evidence which suggests that the local respiratory tract secretory antibody response possesses some degree of immunologic memory. Previous attempts by others to demonstrate this phenomenon have failed (Buscho, Perkins and Wetherbee).

Visualization of rhinoviruses was facilitated by addition of antibody to virus specimens under study. This technique of immune electron microscopy led to the recognition of 2 distinct populations of rhinoviruses--those with empty capsids and those which represented full virions. Empty capsids aggregated with each other as did the full virions indicating a difference in surface antigenic structure /Kapikian and Almeida (London)/.

Coronaviruses. Two human coronavirus serotypes were found to have an *in vitro* shut-off temperature of 35-36°C. This temperature limitation may explain why these viruses apparently produce disease only in the cool passages of the upper respiratory tract. We have consistently been unable to associate these agents with serious lower respiratory tract illness. The coronaviruses appear to have a genome size similar to influenza A virus as indicated by rate of inactivation by UV. A hemadsorption technique for measuring antibody to the OC 43 virus was successfully developed in the past year. Finally, the technique of immune electron microscopy was applied to the coronaviruses and was found to facilitate visualization of these viruses. This important advance will be applied to the search for new coronaviruses during the next year (Kapikian and Bucknall).

Resolution of respiratory tract infection. A new program was instituted this year to evaluate the importance of various host defense mechanisms in recovery from viral infection of the respiratory tract. A non-lethal dose of cyclophosphamide was given to rabbits one day after intranasal inoculation of type 3 parainfluenza virus. Under these conditions the white blood cells were not depressed. Drug-treated rabbits exhibited an altered response to infection--virus persisted in the lungs at high titer for a prolonged period, development of serum antibody was delayed and pulmonary pathology was exaggerated. This type of more severe infection in drug-treated animals was not accompanied by a depression of interferon, suggesting that immunological factors play a major role in recovery from type 3 parainfluenza virus infection (Chalhub and Murphy).

Serial No. NIAID 66A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Epidemiologic Studies of Infections and Illnesses, and Their Control in Junior Village Children

Previous Serial No.: 66A-B

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigators: Dr. Robert M. Chanock
Dr. Neil R. Blacklow
Dr. Reginald H. Mitchell - Dept. of Public Health
District of Columbia
Mrs. Ruth A. Shvedoff
Mrs. C. Eleanor Stewart

Cooperating Units: None

Contract Support:

Contract Project Number	Project Officer (niaid)	Contractor	Contractor's Project Dir.	Annual Fundin Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total	13/12			13/12
Professional	2/12			2/12
Other:	11/12			11/12

Project Description:

This was a long term, intensive study of the illness and microbial experiences of nursery children at Junior Village, a District of Columbia Welfare Institution. This study began in July of 1955 and the field portion of the study was discontinued on May 21, 1969.

Basic Objectives:

The general objective was to maintain under observation a population group suitable for epidemiologic study of occurrence of infections and disease and, in addition, to study host-parasite-disease relationships as they occur naturally and as they could be altered by vaccines and/or chemo-prophylactic agents. A concerted effort was made to (a) find new microbiologic agents which cause disease; (b) find methods of identification of these agents; (c) define modes of spread of infectious agents; and (d) find methods for prevention and treatment of acute illnesses, particularly respiratory illnesses.

Methods Employed and Patient Material:

The field portion of this study was discontinued on May 21, 1969. The epidemiologic setting was described in detail in previous annual reports. Currently, original throat swab specimens from the study are stored at -70°C for the study of coronaviruses when suitable methods for their propagation become available; in addition, anal swab specimens are stored at -20°C for the study of gastroenteritis agents when suitable methods for their propagation are found.

Major Findings:

Epidemiology of Rhinovirus Infection and Characterization of Rhinoviruses- Most respiratory viruses have been studied extensively at Junior Village. However, one major group which has not been studied extensively has been the rhinoviruses since antisera were not available for many serotypes and as a result typing of strains on a limited basis was generally unsuccessful. However, with the completion of the second phase of the rhinovirus collaborative program and the availability of antisera to all 89 serotypes and the one subtype, efforts will be made to study the epidemiology of the numerous rhinovirus serotypes at Junior Village. Thus far, acid lability tests have been carried out on 344 viral isolates from 222 residents and 122 viruses have been found to be acid labile. These studies will be continued in an attempt to determine the epidemiologic pattern of rhinovirus infection in this population.

An Outbreak of Infectious Lymphocytosis - This outbreak occurred in a four-month period when at least 22 cases of acute infectious lymphocytosis were discovered. The leukocyte counts varied from 20,000 to 94,000 per mm. with a relative lymphocytosis on the average of approximately 80%. Routine viral, bacterial, fungal, and parasitic studies were performed on specimens obtained during the outbreak and no definitive etiologic agent was found. Renewed attempts will be made to isolate an etiologic agent by using newer techniques such as organ cultures and experimental cell cultures. The occurrence of EB virus infection in 10 children with infectious lymphocytosis was studied using the immunofluorescent technique. These studies which were reported in last year's annual report revealed that none of 6 children without pre-existing antibody, and none of 4 with such antibody developed four-fold or greater EB virus antibody rises. In addition, paired sera from 9 of the 10 children were tested by the CF technique for evidence of infection with cytomegalovirus (CMV) and herpes simplex virus (HSV); no rises to CMV and one to HSV were detected.

Seroepidemiological Studies of Coronavirus Infections - Paired sera obtained from children admitted to Junior Village between October 15 and December 15 during the years 1961 through 1968 were tested for CF antibody rises to coronavirus strains OC38 and 229E. This period was chosen in an attempt to bracket the winter seasons with a serum collected on admission to the cottage and a serum taken several months later since it was shown previously that adult coronavirus infections were prevalent primarily during the winter. It was noteworthy that 23 (16%) of the 148 children developed significant CF antibody rises to OC38 virus but only 1 of 149 children developed such a rise to 229E virus. In addition 47 (32%) of the children possessed OC38 CF antibody in one or both of the 2 sera tested while only 2 possessed such antibody to 229E virus. Further seroepidemiological studies will be carried out to help elucidate the pattern of coronavirus infection in this population.

Significance of Biomedical Research and the Program to the Institute:

This project represents a systemic approach to determining the various etiologies of acute febrile illnesses. Since carefully gathered epidemiological data and clinical specimens are available, this project also offers an unusual opportunity for the study of the epidemiologic patterns of infections associated with newly discovered agents.

Proposed Course of Project:

The field portion of this study was discontinued on May 21, 1969, when it became apparent that vaccine studies could not be carried out in this population. Studies with specimens obtained from this project will be continued as long as they are profitable.

Honors and Awards:

Recipient of the Meritorious Service Medal, 1970.

Publications:

Blacklow, N.R. and Kapikian, A.Z.: Serologic Studies with EB Virus in Infectious Lymphocytosis. Nature 226: 647, 1970.

Serial No. NIAID 66F
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Attempts to Find the Etiologic Agents of Myocarditis

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Cooperating Units: Dr. Jack Segal and Dr. W. Proctor Harvey, Georgetown University Medical School, Department of Medicine
Mr. L. Cline, Serology Unit, NIAID

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1/12			1/12
Professional:	<1/12			<1/12
Other:	1/12			1/12

Project Description

The basic objective of this study was to attempt to elucidate by epidemiologic methods the etiologic agents involved in the pathogenesis of myocarditis. Although myocarditis patients tended to have a somewhat higher prevalence of CF antibodies than did certain other patients to a few enteroviruses, these differences were not statistically significant. This project is to be discontinued until epidemiological information indicates that further testing would be profitable.

Serial No. NIAID 66G
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Possible Relationship of Viruses with Systemic Lupus Erythematosis

Previous Serial Number: Same

Principal Investigator: Dr. Neil R. Blacklow

Cooperating Units: Dr. Theodore Pincus, Laboratory of Pathology, NCI
Dr. Phillip Grimley, Laboratory of Pathology, NCI
Dr. Alfred Steinberg, Arthritis and Rheumatism Branch, NIAMD

Contract Support:

Contract Project Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Fundin Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	27/12			27/12
Professional:	15/12			15/12
Other:	12/12			12/12

Project Description:

Objectives: To ascertain the possible relationship of viruses with systemic lupus erythematosis (SLE).

Methods Employed:

A. Immunofluorescent Studies of SLE Kidneys - Electron microscopists from several laboratories have recently reported the presence of virus-like particles in tissue sections of kidneys from patients with SLE. The morphology of these particles suggests that they may be myxoviruses or paramyxoviruses. Whether these particles are viruses is unknown. However, there is precedent in mice for virus-induced renal disease by the mechanism of virus-antibody immune complexes deposited in renal glomeruli. Accordingly, we have undertaken a program to search for viruses in the kidneys and other tissues of SLE patients.

Renal biopsies from SLE patients have been obtained through the courtesy of Drs. John Decker and Norman Talal, Arthritis and Rheumatism Branch, NIAMD. The biopsies are frozen, sectioned and then reacted in direct immunofluorescence tests (FA) with fluorescein isothiocyante conjugated animal antisera against all of the human myxoviruses, paramyxoviruses, herpes simplex, and cytomegalovirus. These antisera have been carefully sectioned on the basis of their strongand specific reactivity against tissue cultures infected with the homologous virus.

B. Cell Cultures Derived from SLE Renal Tissue - Renal biopsy material is minced, trypsinized and then cultured according to standard procedures. Cell lines, primarily fibroblastic, are then established and carried through serial passages. The cell lines are studied for the presence of viral agents by observation for cytopathic changes, by hemadsorption tests with guinea pig and rhesus erythrocytes, and by direct FA tests. Fluids from the cell cultures are tested in CF for the presence of viral antigens.

C. Tissue Culture Co-cultivation Studies - The kidney cell lines are passaged (at an early passage level) with each of the following cells: HeLa, BSC-1, Rhesus monkey kidney and HEK. These co-cultivated cell mixtures are then tested for the presence of viral agents by the techniques outlined in section B.

D. Serologic Studies - Sera from patients with SLE are tested in CF for reactivity with human viral antigens and with control cellular antigens used in the preparation of viral antigens.

Major Findings:

A. Studies of SLE Renal Tissue - Five open renal biopsies of patients with active SLE have been studied by electron microscopy, immunofluorescence (FA) and tissue culture. Three of the 5 biopsy specimens examined by electron microscopy contained the virus-like inclusions in the glomerular endothelium described by others. These structures have been identified in a variety of clinical diseases in addition to SLE, and appear to be considerably larger than viruses of the myxo-paramyxovirus group. They may possibly be regarded as a unitary modification of the endoplasmic reticulum which develops in transitional forms in response to various insults.

Results of RA studies of the 5 SLE renal biopsy specimens were negative with most labelled antiviral sera. Positive glomerular staining was seen in isolated instances, but showed no correlation between the microtubular structures and a specific viral antigen.

Co-cultivation studies of tissue cultures derived from the 5 SLE kidneys have failed to reveal the presence of a virus: cytopathic effects were absent, hemadsorption, FA and CF tests have been negative.

B. Serologic Studies - We have tested sera of SLE patients and of matched control subjects in CF against several RNA and DNA viral antigens. Elevated titers against nearly all viral antigens have been seen with SLE sera compared to sera of matched control patients. Further, there has been no correlation of elevated SLE serum titers with any specific virus group. In addition, 40% of SLE sera have reacted with non-virus containing control antigen preparations of human, monkey or hamster origin. This finding, presumably due to the abnormal reactivity of SLE sera with nuclear and cytoplasmic components, reflects the complexity of viral immunologic studies in SLE.

Significance of Program to NIH

The etiology of human "auto-immune" diseases is unknown. There is suggestive evidence that viral agents may be involved in some direct or indirect way in the pathogenesis of these diseases, particularly SLE. Efforts to search for viruses in these diseases appear to be indicated, in view of (a) the importance of these diseases to our society and (b) the clues from animal experiments and studies of human SLE kidneys that viruses may play a role in these diseases.

Proposed Course of Project:

At this point, there is little evidence to implicate any one specific virus with SLE. The possible relationship of viruses to SLE, if at all, presently appears to be quite complex. Proposed future studies will be limited to analysis of altered immunologic reactivity of SLE seraagainst viral antigens and their cellular controls.

Honors and Awards: None

Publications:

Pincus, T., Blacklow, N.R., Grimley, P.M., and Bellanti, J.A.: "Glomerular Microtubules of Systemic Lupus Erythematosus", The Lancet, November 21, 1970, 1058-1061.

Serial No. NIAID 66H
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Etiology of Acute Infectious Non-bacterial Gastroenteritis

Previous Serial Number: Same

Principal Investigators:
Dr. Neil R. Blacklow
Dr. Robert F. Buscho
Dr. Robert M. Chanock
Dr. Raphael Dolin

Cooperating Units:
Dr. David Fedson and Dr. Julius A. Kasel, Medical Virology Section, Laboratory of Clinical Investigation, NIAID
Dr. Herbert DuPont and Dr. Richard Hornick University of Maryland Medical School

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	27/12			27/12
Professional:	15/12			15/12
Other:	12/12			12/12

Project Description:

Objectives: (1) To search for viruses which may play an etiologic role in the syndrome of acute infectious non-bacterial gastroenteritis; (2) to characterize any new viral agents that may be discovered, by study of their biologic, immunologic, and epidemiologic properties; (3) to reproduce the syndrome in experimentally infected susceptible adults, and to study the clinical course of the syndrome as well as the nature of the immunologic response(s) to infection; (4) to cultivate the agents of acute infectious non-bacterial gastroenteritis in tissue culture or organ culture in vitro.

Methods Employed:

A. Intestinal Organ Cultures - Because of the evidence that occult respiratory viruses can be detected using tracheal organ culture systems, we have applied a similar approach, employing human fetal intestinal organ cultures in the study of non-bacterial gastroenteritis. Prior to our studies, there have been no known attempts to propagate the agent(s) of non-bacterial gastroenteritis in intestinal organ culture. Further, there have been no reports that indicate successful maintenance of human intestinal organ cultures beyond 24 hours. We feel that organ cultures may provide a sensitive system for the replication and detection of heretofore occult viral agents.

We have established small intestinal organ cultures from human fetuses obtained at hysterotomy or hysterectomy. These fetuses are usually from 12 to 20 weeks gestation. Fetuses from therapeutic abortions, performe for psychiatric reasons, are obtained from several regional hospitals; the patients are free of apparent infectious diseases at the time of hysterotomy or hysterectomy. The technique for our establishment of intestinal organ cultures was described during 1970.

B. Other Culture Systems Employed - Although the principal culture system employed has been the organ culture, we have attempted to isolate viral agents from clinical specimens with the use of a variety of primary and continuous tissue cultures. Fluids from these inoculated tissue cultures are serially blind passaged to fresh cultures which are then observed for the presence of cytopathic effects, hemadsorption, and FA staining. In addition, fluids from inoculated organ cultures are passaged into tissue cultures.

C. Source of Clinical Material - Stool and paired serum specimens have been collected from a variety of outbreaks of non-bacterial gastroenteritis. Particular effort has been directed at obtaining specimens from individuals who appear to represent "secondary attack" cases. Specimens have been obtained from (a) outbreaks at Norwalk, Ohio, Tampa, Florida, and Denver, Colorado, studied extensively by the Center for Disease Control (courtesy of Drs. M. Hatch and F. Judson, CDC); (b) outbreaks among families of NIH personnel; (c) an outbreak at Henrytown State Hospital near Westminster, Maryland (courtesy of Maryland Department of Health).

D. Preparation of Inoculae Derived from Clinical Material - Stool specimens are suspended in veal infusion broth with 0.5% albumin, clarified by low-speed centrifugation, and then filtered through a 1200 mμ membrane to exclude bacteria. Antibiotics are then added to the filtrate which is then dispensed into vials and frozen at -70°C. These inoculae are then available for use in (a) inoculation of organ cultures, (b) inoculation of tissue cultures, and (c) experimental infection of human volunteers, after extensive safety tests to rule out detectable infectious agents.

E. Volunteer Studies - Over the past 24 years, a number of laboratory groups have induced "gastroenteritis" in over 50 adult volunteers by administering stool filtrates and supernates by a variety of techniques. There have been no reports of apparent complication, or sustained morbidity beyond that of the self-limited, spontaneously resolving acute illness. Reports of experimentally produced illness have included those by Reimann et al. (J.A.M.A. 127: 1, 1945), Jordan et al. (J. Exp. Med., 461, 1953), Fukumi et al. (Japanese J. Med. Sci., and Pathol. 10: 1, 1957). The work of Jordan et al. demonstrated the existence of 2 similar, though clinically distinct, syndromes derived from 2 separate outbreaks of gastroenteritis, with each inoculum able to produce short term homologous but not heterologous immunity. Unfortunately none of these materials were preserved so that the agents described by Jordan and other workers are no longer available.

During the past year, we have conducted 8 volunteer studies in order to ascertain with certainty the presence and nature of a transmissible material. These studies have been conducted both at the Maryland House of Correction and at the Clinical Center. These studies have already yielded considerable information about the infectivity of several inocula and have provided a description of the biologic properties of the etiologic agent of the Norwalk, Ohio outbreak.

Major Findings:

A. Transmission of Acute Infectious Non-Bacterial Gastroenteritis to Volunteers - We have serially transmitted acute non-bacterial gastroenteritis through 3 human passages by the oral administration of fecal filtrates derived originally from the Norwalk, Ohio outbreak. A spectrum of clinical responses has been noted, ranging from a predominantly diarrheal disease without vomiting to an illness characterized by vomiting without accompanying diarrhea. As a result of various treatments of disease-producing inocula, we have defined certain properties of the etiologic agent, using volunteers as the experimental host: (1) ether treated inoculum caused disease in 3 of 5 volunteers; (2) inoculum filtered through a 110 mm gradacol membrane has induced disease in 2 of 9 subjects; a 60 mm gradacol filtrate produced illness in 1 of 9 men; (3) 5 volunteers who had previously experienced induced illness were rechallenged with the Norwalk inoculum and all remained clinically well; (4) the inoculum produced disease at a 10^{-2} dilution but not at a 10^{-4} dilution; (5) acid (pH 2.5) treated inoculum caused disease in 2 of 3 men; (6) nasopharyngeal washings obtained from an ill volunteer did not induce illness when administered orally to 3 men.

The disease producing Norwalk inoculum is free from aerobic and anaerobic bacteria. Further, it does not possess bacterial enterotoxins as determined by rabbit ileal loop assay (courtesy of Dr. S. Formal, Walter Reed). The resulting dilution of original rectal swab material that has occurred through 3 human passages makes it highly improbable that a non-replicating agent, such as a toxin, has served as the causative agent. As a result of our studies, the etiologic agent of the Norwalk outbreak appears likely to be a non-enveloped, acid stable, relatively small virus, which confers at least short term immunity to reinfection.

Clinical studies of intestinal absorptive properties have been performed on volunteers who have received the Norwalk inoculum. Abnormal xylose tolerance tests were demonstrated at the time of illness in each of the 5 volunteers studied who experienced induced illness, and in 1 of 3 volunteers who were administered the Norwalk inoculum but who did not become ill. There is, therefore, evidence for a pathophysiologic alteration in small intestinal function during experimental non-bacterial gastroenteritis.

B. Studies with Intestinal Organ Cultures and Other Culture Systems - We have established small intestinal organ cultures from human fetal material, and have maintained these cultures for up to 21 days. These cultures have supported the growth of a variety of known viral agents; some of these viruses produce cytopathic alteration of the organ cultures, and others do not. Stool inocula from several outbreaks, including the Norwalk Outbreak, have been inoculated into organ culture and passaged. Gross cytoarchitectural changes have not been observed with these latter inocula.

The Norwalk inoculum was passaged 3 times in organ culture, and the third passage material induced gastroenteritis in 1 of 4 volunteers. Since the dilution of the original inoculum through 3 passages was only $10^{4.9}$, disease could have been caused by persistence of an agent from a high-titered inoculum. Fluids collected from a passage of the inoculum to a 10^{-14} dilution failed to produce disease in 4 volunteers.

At this point, studies utilizing a variety of primary and continuous tissue cultures, immunofluorescent techniques, and electron microscopy have offered no conclusive leads to a laboratory assay system for the Norwalk agent.

Significance of Program to NIH

Acute infectious non-bacterial gastroenteritis is a major widespread infectious disease of uncertain etiology that affects a significant proportion of the American pediatric and adult population. Identificatio of etiologic agent(s) represents an initial step towards the ultimate goals of prevention and therapy. The newly developed organ culture technique, as well as the volunteer studies, should produce useful information on the etiology, epidemiology, and pathogenesis of this disease.

Proposed Course of Project

The search will continue for the detection of etiologic agents from outbreaks of non-bacterial gastroenteritis. Development of sensitive assay techniques to detect the replication of agents in the intestinal organ cultures will continue. Any new viral agents that may be discovered will be studied with respect to their biologic, immunologic, and epidemiologic properties. Attempts will be made to transmit the illness to volunteers from outbreaks other than the Norwalk epidemic, and to characterize these agent(s) in the laboratory and in volunteers. Any new inocula that produce experimental illness will be tested in volunteers for cross-protective properties with the Norwalk agent.

Honors and Awards:

Lecture: "Viral Diarrhea", Drs. Blacklow and Dolin, University of Maryland Medical School, February 25, 1971

Publications:

Dolin, R., Blacklow, N.R., DuPont, H., Formal, S., Buscho, R.F., Kasel, J.A., Chames, R.P., Hornick, R. and Chanock, R.M.: Transmission of Acute Infectious Non-Bacterial Gastroenteritis to Volunteers by Oral Administration of Stool Filtrates, J. Infect. Dis. 123, 307-312, 1971.

Dolin, R., Blacklow, N.R., Malmgren, R.A., and Chanock, R.M.: Establishment of human fetal intestinal organ cultures for growth of viruses. J. Infect. Dis. 122: 227-231, 1970.

Serial No. NIAID 66I
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Natural History and Defective Nature of Infection with Adenovirus-Associated Viruses (AAV)

Previous Serial Number: Same

Principal Investigator: Dr. Neil R. Blacklow

Other Investigators: Dr. M. David Hoggan, Laboratory of Viral Diseases
Dr. Raphael Dolin, Laboratory of Infectious Diseases

Cooperating Units: Dr. Robert H. Parrott, Children's Hospital
Washington, D.C.

Contract Support:

Contract Project Number	Project Off. (NIAID)	Contractor	Contractor's Project Dir.	Annual Fundin Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	18/12			18/12
Professional:	6/12			6/12
Other:	12/12			12/12

Project Description:

Objectives: (1) To study the biological and immunological properties of the defective AAV, including the mechanisms of their relationships with adenoviruses and herpesviruses; (2) to study the natural history of AAV infection, including the epidemiology of AAV infection in man and the possible participation of AAV in the disease response to adenovirus and herpesvirus infections; (3) to investigate the biologic and immunologic relationships between AAV and other members of the parvovirus group.

Methods Employed:

A. Cell Cultures - The AAV are small defective DNA viruses that are able to replicate completely only in cell cultures coinfected with a helper adenovirus. For this purpose, a variety of cell cultures are employed, particularly human embryonic kidney and HEP-2 cells.

B. Assay Methods - Because the AAV do not produce cytopathic effects in tissue cultures, they are assayed primarily by immunologic techniques. Accordingly, AAV-specific antigens are readily detected by complement-fixation (CF) and immunofluorescence (FA) tests. These CF and FA assays make possible neutralization and infectivity titration tests in the study of AAV.

C. Biophysical Techniques - Purified AAV, free of detectable adenovirus, is prepared by the isopycnic density gradient centrifugation technique. AAV prepared in this manner is then used in CF to detect AAV CF antibodies in man. The purified AAV is also used to hyperimmunize guinea pigs. Purification of other parvoviruses (H-1, Rat Virus, Densonucleosis Virus, Haden Virus, and Minute Virus of Mice) has been conducted with the same methods.

Major Findings:

A. Epidemiology of AAV Infection in Man - The AAV are a completely unique phenomenon in animal virology--a virus which appears to be absolutely dependent on another virus for its maintenance in nature. We have isolated AAV types 2 and 3 from children in the Junior Village population, and an intensive study of these children during an 8-week period has shown that AAV can spread in epidemic proportions. All AAV isolates have been in association with adenovirus isolates, suggesting a close relationship *in vivo* between AAV and denovirus. The extent of AAV infection in man appears to be widespread, inasmuch as 50 to 70 percent of young adults possess neutralizing antibodies against AAV types 1, 2, and 3. Whether AAV has a role in acute or chronic human disease is currently unknown; there was, however, no correlation between acute febrile illness and recovery of AAV at Junior Village.

During the past year, we have conducted a serologic study of AAV infection over an 8 year period at Children's Hospital, D.C. We have studied 3 categories of children: (1) children with respiratory tract disease temporally associated with adenovirus infection; (2) children infected with adenovirus but who were free from infectious disease; (3) asymptomatic children who were not adenovirus-infected. A significantly greater number of children with adenovirus-associated respiratory illness developed CF antibodies to AAV types 2 and 3 than did children in either of the other two categories. This finding suggests that extensive adenovirus infection is necessary for detectable AAV infection to occur, and that AAV infection in man does not lessen the severity of disease produced by adenovirus. Seroconversion to AAV was not associated with any one form of clinical illness.

B. Enhancement of AAV by Human Herpesviruses - We have previously studied the mechanisms by which adenovirus aids in the replication of AAV in tissue culture. Cells are rendered competent to synthesize AAV antigens as well as infectious AAV only when they are coinfected with an adenovirus. Early events in the adenovirus replicative cycle are not sufficient to aid in the formation of infectious AAV; further, adenovirus DNA synthesis is a necessary event if infectious AAV is to be produced.

Recently we have shown that s second group of animal viruses, the herpesviruses, are able to provide a limited helper function for AAV. The human herpesviruses (EB virus, cytomegalovirus, varicella and herpes simplex) provide necessary function(s) for AAV to form immunofluorescent-stainable antigen but not infectious virus or particles visible by electron microscopy. Therefore, human herpesviruses provide incomplete helper function(s) for the growth of AAV as contrasted with adenoviruses which provide all the required functions for a complete helper effect. The new findings with the human herpesviruses now raise the possibility that AAV may participate in the disease response to herpesvirus infections as well as to adenovirus infections.

We have analyzed some of the sequential events that are provided by herpes simplex for the enhancement of AAV. In contrast with adenovirus helper, newly synthesized infectious herpes simplex is not needed for the formation of AAV FA antigen; this has been shown by both growth cycle and arginine deprevation studies. Further, these studies indicate that the helper effect of herpes is temporally associated with the replication of herpes DNA. Studies utilizing the DNA-inhibitor cytosine arabinoside, also indicate that de novo DNA synthesis in coinfected cells is required for AAV FA antigen formation.

C. Relationships Between AAV and Other Parvoviruses - In collaboration with Dr. Hoggan, LVD, NIAID, we have initiated comparative studies of several candidate parvoviruses. These include AAV types 1 through 4, the rodent parvoviruses (H-1, Rat Virus, Minute Virus of Mice), Haden virus (hemadsorbing enteric virus of calves) and Densonucleosis virus (of the insect Galleria mellonella). These viruses have been purified and banded in CSC-1; they band at densities of 1.38-1.46 gm/cm^3. Hyperimmune guinea pig sera have been prepared, and then used to study the immunologic relatedness of these viruses. These studies are currently in progress; preliminary data indicate immunologic relatedness among the rodent parvoviruses as measured by immunofluorescence.

Significance of the Program to the Institute:

The importance of AAV in human disease is unknown, but it is clear that these viruses commonly infect man. They are a unique phenomenon in that they appear to be dependent on another virus for their maintenance in nature. Because of the pathogenic and potential oncogenic effects of adenoviruses and herpesviruses, it is important to clarify the clinical importance of AAV infections. The AAV do have the ability to lie dormant in tissue culture awaiting adenovirus to become manifest. They provide a unique model in which to study a defective virus that requires another virus for replication in man as well as in tissue culture

Proposed Course of Project:

Efforts will continue to assess the potential role of AAV in human disease acting either by itself or in concert with its helper virus. Studies will also continue on the mechanism of the herpesvirus-AAV relationship, as well as on the inter-relationships among the parvoviruses.

Honors and Awards: None

Publication:

Blacklow, N.R., Hoggan, M.D., and McClanahan, M.S.: Adenovirus-Associated viruses: Enhancement of human herpesviruses. Proc. Soc. Exp. Biol. Med. 134: 952-954, 1970.

Blacklow, N.R., Dolin, R. and Hoggan, M.D.: Studies of the Enhancement of an Adenovirus-associated Virus by Herpes Simplex Virus. J. Gen. Virol. 10: 29-36, 1971.

Blacklow, N.R., Hoggan, M.D., Sereno, M.S., Brandt, C., Kim, H.W., Parrott, R. and Chanock, R.M.: A Seroepidemiologic Study of Adenovirus-associated Virus Infection in Infants and Children. Amer. J. Epidem. In press, 1971.

Serial No. NIAID 69A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Non-bacterial Respiratory Disease
A. Children

Previous Serial Number: Same

Principal Investigator: Dr. Robert M. Chanock

Other Investigator: Dr. Albert Z. Kapikian

Cooperating Units: Dr. R.H. Parrott, Children's Hospital Research Foundation, Washington, D.C.

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 135,840
PH 43-62-473	Dr. Chanock	Children's Hosp. of D.C.	Dr. Parrott	143,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	36/12			36/12
Professional:	12/12			12/12
Other:	24/12			24/12

Project Description:

Objectives: (1) To search for new agents responsible for respiratory disease in infants and children; (2) to delineate the epidemiology and pathogenesis of viruses which have been recently associated with respiratory disease; (3) to determine the major determinants of host resistance to infection and illness; (4) to continue the surveillance of viruses previously established as playing a role in respiratory disease, i.e., the epidemiology and contribution to overall respiratory disease experience during infancy and childhood; (5) to evaluate experimental vaccines for safety, antigenicity, and effectiveness against naturally occurring respiratory tract disease.

Methods Employed:

Infants and children with respiratory disease and suitable control subjects without such disease are studied at the Children's Hospital, District of Columbia. The main emphasis is on the use of various tissue culture systems and artificial media for the recovery of viruses and mycoplasmas. All specimens from the patient are inoculated directly into tissue cultures and onto mycoplasma media without prior freezing and thawing, if feasible. In addition to specimens for isolation of viruses and mycoplasma, serial nasal washings are taken to study respiratory tract secretory antibody response.

After the various agents have been identified, their contribution to the different respiratory disease syndromes is estimated by comparing the recovery rate from such groups with that observed for healthy children free of respiratory symptoms. In addition, children with severe respiratory disease admitted to the hospital as well as comparable control subjects, are studied serologically for evidence of infection with the known respiratory viruses and mycoplasmas as well as any new agents which might emerge as potentially important pathogens during the course of the study.

Respiratory viruses which are attenuated by exposure to a chemical mutagen and which exhibit no virulence for adult volunteers are administered to children under carefully controlled conditions in the isolation unit of the Children's Hospital Clinical Research Center. As in other studies, parental consent is obtained in each instance. Vaccinees are observed closely for evidence of illness and specimens are taken for the study of virus excretion pattern, serum antibody response and local secretory antibody response.

Major Findings:

(1) Nasal Secretory Antibody for RS Virus - In previous studies we had shown that approximately 40% of infants and young children who were admitted to the hospital with RS virus pneumonia or bronchiolitis developed a rise in titer of neutralizing activity for this virus in their nasal secretions. In the other 60% of patients we failed to detect a response because the acute phase secretion contained as high a level of neutralizing activity as the convalescent specimen. Initially we attributed this phenomenon to the early development of a local antibody response; we postulated that local antibody had been produced in an appreciable proportion of patients prior to admission to the hospital. Another possibility, which was not highly regarded at the start, involved a non-antibody inhibitor which inactivated virus. Recent work suggests that the latter explanation is correct and that an inhibitor is responsible for obscurring the development of a local antibody response in a significant proportion of young patients with RS virus disease.

Inhibitory activity was detected in most nasal secretions collected from young infants born after the last RS virus epidemic. There was very little opportunity for these infants to have been infected with RS virus and for this reason the neutralizing activity in their secretions appeared to be non-specific. The behavior of the inhibitor in a sucrose gradient was studied in order to obtain preliminary information about its physical state. RS virus neutralizing activity in secretions from adults in most instances sediments as an 11S moeity and has the properties of a dimeric 11S IgA. This was confirmed in the current study. In contrast, most of the secretions from normal young infants (who should not have been infected previously with RS virus) or infants in the acute phase of RS disease contained inhibitory material which sedimented in the lower portion of the gradient with a sedimentation coefficient greater than 11S; these heavy fractions with inhibitory activity did not contain detectable IgM. During convalescence from RS disease neutralizing activity in secretions of young infants sedimented less rapidly and exhibited a major peak in the 11S region of the sucrose gradient. This suggests that specific antibody was present in the convalescent secretions. At present we know little about the inhibitor in normal secretions other than that it sediments more rapidly than specific 11S IgA antibody. It is of interest that following infection with RS virus there appears to be a decrease in the rapidly sedimenting inhibitor and this is associated in time with the emergence of 11S neutralizing activity, presumably specific antibody.

Although the normal inhibitor tends to obscure the specific local antibody response to RS it is nevertheless possible to detect such a response in a significant proportion of infected individuals. Additional evidence for the occurrence and specificity of the local antibody response was sought in a prospective study of children who were investigated before and after the community wide RS virus epidemic of 1969-1970. Sixty children were kept under surveillance and tested for RS virus at the time of illness. In addition serum and nasal secretions were collected before and after the winter outbreak of RS virus infection. Twenty-two children developed a rise in nasal secretion neutralizing activity during the period which spanned the epidemic. Sixty percent of these children developed other evidence of RS infection, such as a rise in serum neutralizing and/or CF antibody. In contrast only 24% of the children who failed to develop a nasal secretion response had a rise in serum neutralizing and/or CF antibody. These findings confirm the results of a similar study performed last year and suggest that the rises in nasal secretion neutralizing activity which were measured were in most instances specific for RS virus. A number of nasal secretion responses could have been missed because there was a long interval between collection of the pre and post epidemic specimens and we have found that secretory antibody usually declines more rapidly than serum neutralizing antibody.

(2) Evaluation of a Temperature Sensitive Mutant of RS Virus as a Potential Vaccine Strain - Special emphasis has been given to the development of a live attenuated strain of RS virus for use in infants because of the increasing evidence that local respiratory tract defenses play a major role in resistance to infection. The ts-1 mutant of RS virus, induced by the chemical mutagen 5-fluorouridine, was chosen for the initial trials on the basis of its behavior in vitro in tissue culture and in vivo in the experimental hamster host and in adult volunteers. This mutant did not produce plaques at 37°C and above. In the hamster infection was limited to the cooler upper respiratory tract (32-34°C) and virus was not found in the lungs (37°C). The mutant was stable in culture and in hamsters, i.e., there was no evidence of reversion to the wild phenotype. The mutant infected adult volunteers without producing disease and induced resistance to subsequent challenge with virulent wild type virus. Isolates obtained from the adult volunteers retained their ts property.

To date 14 children have been given the ts-1 mutant. Six were 4-6 years old, 6 were 2-4 years old, and 4 were 1-2 years old. Thirteen of the 14 children became infected, but respiratory illness did not occur. In the 4-6 year group, 2 children shed virus and 4 developed a rise in nasal secretion antibody. Virus was shed by each of the 10 younger children. Thus far 20 isolates from 6 of the children who shed virus have been analyzed for evidence of reversion. All but 2 of the isolates retained the ts property. The 2 wild type viruses were recovered from an 18 month old child. These isolates produced plaques with high efficiency at 39°C, a temperature which was completely restrictive for the ts-1 mutant. The pattern of shedding of virus by this child was unusual in that the first isolate on day 7 contained wild type virus; the isolates on days 8 and 9 were ts, while the last isolation on day 19 contained wild type virus. This pattern is difficult to interpret. Although the child was kept in relative isolation in the Clinical Research Center at Children's Hospital of District of Columbia, one cannot rule out the possibility of hospital acquired super-infection with naturally occurring wild type virus. Since this is unlikely, we must tentatively conclude that reversion occurs in a small minority of infections. In this instance of the 18 month old child, who apparently shed revertant virus, the pattern of shedding suggests that revertant virus did not take over and rapidly replace the ts mutant; revertant virus was detected on day 7, but only ts virus was recovered the next 2 days. In any case the sporadic shedding of apparent revertant virus was not associated with respiratory tract disease.

(3) Epidemiology of M. pneumoniae Infection in Infants and Young Children - There is an unusual age distribution of M. pneumoniae illness in which disease occurs with highest frequency between 5 to 15 years of age, while illness is infrequent below 5 years of age. During the ongoing study at Children's Hospital of D.C. there was an unexpectedly high frequency of shedding of M. pneumoniae from young

children who were not generally subject to the illness producing effects of the organism. Recent studies utilizing the highly sensitive complement dependent mycoplasmacidal antibody test revealed that most 1-2 year old children possessed such antibody, whereas infants 7-12 months of age had this antibody less often. These serologic findings support the view that M. pneumoniae infection (or stimulation with a cross reactive antigen) occurs commonly during early life. Further the findings suggest the possibility that disease may be favored by prior sensitization induced by a silent infection sustained at an early age.

Significance of the Program to the Institute:

Respiratory disease is the most common ailment of man, particularly during infancy and early childhood when many of the most serious expressions of such illness occur. Detailed knowledge of the agents responsible for respiratory tract disease and an understanding of their natural history are necessary before effective prevention can be achieved.

The insight that we have gained into the pathogenesis, natural history and immunologic determinants of resistance in RS virus disease has immediate significance to the Institute's program for vaccine development. In this context the role of local respiratory tract antibody in resistance to RS infection and illness assumes increasing importance both in our understanding of respiratory disease mechanisms and in our attempts to develop effective immunoprophylaxis. We must have detailed information concerning the nature of the respiratory tract secretory antibody response to RS virus infection, the temporal course of this response and the level of local antibody which provides protection before meaningful standards can be established for experimental vaccines.

One of the most pressing needs in respiratory disease control is the prevention of serious RS virus illness during infancy. The present efforts at immunoprophylaxis utilizing the ts-1 mutant of RS virus are promising and are marred only by the recent evidence which suggests that reversion to wild phenotype may occur occasionally in vivo. The full significance of this phenomenon awaits the results of more extensive testing of the ts-1 mutant. Thus far, infection of young children has been silent and thus it seems justified to proceed with evaluation of the mutant.

The serologic findings which suggest frequent inapparent infection with M. pneumoniae in infants and children under 5 years of age are intriguing. Possibly the occurrence of disease at a later date may result from second infection in a sensitized host. The age distribution of M. pneumoniae pneumonia is consistent with this possibility.

Proposed Course of Project:

It is planned that this study will continue for a period of years since a large segment of respiratory disease in infancy and childhood remains to be explained--approximately 40%. In addition, viruses responsible for respiratory illness vary in different times and in different localities; therefore, surveillance should continue in order to comprehend the larger picture. Efforts to increase our understanding of the pathogenesis of viral and mycoplasmal lower respiratory tract disease of infancy and childhood will continue. Particular attention will be given to elucidating the possible role of sensitization in the pathogenesis of *M. pneumoniae* pneumonia.

The ts-1 mutant of RS virus will be evaluated in infants pending successful completion of current studies in 1-2 year old children. Initially infants 7-12 months of age who are free of maternally derived serum antibody will be given the mutant under carefully controlled conditions. If infection in this age group of seronegative individuals occurs without symptoms the mutant will then be tested in young infants with maternally derived antibody. Special attention will be given to the question of reversion and all isolates will be characterized for the ts property.

Honors and Awards:

Director International Reference Laboratory for Respiratory Viruses other than Influenza, World Health Organization

Director International Reference Laboratory for Mycoplasmas, World Health Organization

Member International Subcommittee on Taxonomy of the Mycoplasmatales, International Committee on Nomenclature of Bacteria of the International Association of Microbiological Socities

Invited speaker Xth International Congress for Microbiology, held in Mexico City, August, 1970. Topic: "Immunological Aspects of Respiratory Infections."

Invited speaker 1970 Scientific Assembly of the Medical Society of D.C., September, 1970. Topic: "Vaccination Against Pediatric Illness."

Member Commission on Acute Respiratory Disease, Armed Forces Epidemiology Board, Washington, D.C. Participated in meeting on A. R.D. and Strep/staph Diseases, October, 1970, Atlanta, Georgia.

Associate Member Commission on Influenza,Armed Forces Epidemiology Board, Washington, D.C. Participated in meeting at Walter Reed Army Medical Center, November, 1970.

Invited participant, Pan American Health Organization/W.H.O. International Conference on Vaccines Against Viral, Rickettsial and Bacterial Diseases of Man, December, 1970, Washington, D.C. Topic: "Vaccines for Respiratory Virus Diseases other than Influenza."

Served on Editorial Boards of American Journal of Epidemiology, Journal of Immunology, and American Journal of Diseases of Children.

Invited speaker University of Minnesota, Dept. of Laboratory Medicine's Grand Rounds, February 26, 1971: Topic: "Prophylaxis of Acute Respiratory Tract Disease."

Recipient of DHEW's Distinguished Service Medal, April 15, 1971.

Invited participant Workshop on "Mycoplasmatales as Agents of Disease, March 29, 1971, at NIH.

Invited participant — 1971 Congress of the South African Society of Pathologists, Johannesburg, June 28, 1971. Topics: "Disease Caused by Mycoplasma pneumoniae" and "Control of Non-Bacterial Respiratory Tract Disease."

Publications:

Bellanti, J.A., Smith, C.B., Kim, H.W., Mills, J., Gerin, J.L., Parrott, R.H., and Chanock, R.M.: Serum and local respiratory antibody responses following infection with Mycoplasma pneumoniae or respiratory syncytial virus. In Dayton, D.H., Jr., Small, P.A., Jr., Chanock, R.M., Kaufman, H.E., and Tomasi, T.B., Jr. (Eds.): The Secretory Immunologic System. Wash., D.C., G.P.O., 1971, pp 183-194.

Chanock, R.M.: Myxovirus para-influenzae et virus respiratoire syncytial virologie. Epidemiologie. Prophylaxie. In Debre, R. and Celers, J.H. (Eds.): Clinique Virologique. Paris, France, Editions Medicales Flammarion, 1970, Chapter VI, pp. 895-915.

Chanock, R.M.: Local antibody and resistance to acute viral respiratory tract disease. In Dayton, D.H., Jr., Small, P.A., Jr., Chanock, R.M., Kaufman, H.E., and Tomasi, T.B., Jr. (Eds.): The Secretory Immunologic System. Wash., D.C., G.P.O., 1971 pp 83-92.

Chanock, R.M.: Control of acute mycoplasmal and viral respiratory tract disease. Science 169: 248-256, 1970.

Chanock, R.M., Kapikian, A.Z., Perkins, J.C., and Parrott, R.H.: Vaccines for non-bacterial respiratory diseases other than influenza. Proceedings of PAHO/WHO Intl. Conference on Application of Vaccines Against Viral, Rickettsial, and Bacterial Diseases of Man, Washington, D.C., December 14-18, 1970. In press, 1971.

Chanock, R.M., Kapikian, A.Z., Mills, J., Kim, H.W., and Parrott, R.H.: Influence of immunological factors in RS virus disease of the lower respiratory tract. Arch. Environ. Health 21: 347-356, 1970.

Chanock, R.M.: Immunological aspects of respiratory virus infections. 10th International Congress for Microbiology (Colloquium on Respiratory Virus Infections), August 9-15, 1970, Mexico. In press, 1971.

Kim, H.W., Arrobio, J.O., Pyles, G., Brandt, C.D., Camargo, E., Chanock, R.M., and Parrott, R.H.: Clinical and immunological response of infants and children to administration of low-temperature-adapted respiratory syncytial virus. Pediatrics, In press, 1971.

Parrott, R.H., Kim, H.W., Brandt, C.D., and Chanock, R.M.: Vaccination against pediatric illness caused by parainfluenza virus, respiratory syncytial virus and certain adenoviruses. Current status and perspective. Med. Ann. D.C. 39: 612-614, 1970.

Parrott, R.H., Kim, H.W., Bellanti, J.A., Arrobio, J.O., Mills, J., Brandt, C.D., and Chanock, R.M.: Development of nasal secretion neutralizing activity in infants and children following natural RS virus infection or administration of cold-adapted RS A2 virus. In Dayton, D.H., Jr., Small, P.A., Jr., Chanock, R.M., Kaufman, H.E., and Tomasi, T.B., Jr. (Eds.): The Secretory Immunologic System. Washington, D.C., G.P.O., 1971, pp. 167-182.

Parrott, R.H., and Chanock, R.M.: Respiratory viruses: Non-bacterial respiratory tract agents. In Cooke, R.E. (Ed.): The Biologic Basis of Pediatric Practice. Volume 1, New York, McGraw Hill Book Co., 1968. pp. 167-182.

Sta. Ana., P.P., Arrobio, J.O., Kim, H.W., Brandt, C.D., Chanock, R.M. and Parrott, R.H.: Serum complement in acute bronchiolitis. Proc. Soc. Exp. Biol. Med. 134: 499-503, 1970.

Serial No. NIAID 69C
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Etiology of Acute Respiratory Disease in Adults

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr. Lee Cline, Serology Unit, NIAID
Dr. James MacLowry, Clinical Center, Clinical Pathology, Infections Laboratory Service
Dr. Wilmer Dockstader, Bacteriology Unit Environmental Services Branch

Contract Support:

Contract Project Number	Project Officer (NIAID)	Contractor	Contractor's Project Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs.	Dr. Gilden	$ 135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	24/12			24/12
Professional:	8/12			8/12
Other:	16/12			16/12

Project Description:

Objectives: (1) To determine the role of known viruses and bacteria as etiologic agents of acute upper respiratory illnesses in adult populations; (2) to find and characterize new agents which may be human respiratory tract pathogens; (3) to investigate the sensitivity of new experimental cell culture systems for the isolation of rhinoviruses and the recently described human coronaviruses.

Methods Employed:

A. National Institutes of Health (NIH) Volunteer Study - The current study began on September 9, 1965 and continues to the present. (An earlier similar study began in 1962 and was terminated in 1964) Employees of NIH with respiratory illnesses volunteer a nasopharyngeal washing, a throat swab, and a blood specimen during the acute phase of a respiratory illness. An additional nasopharyngeal washing and blood specimen are obtained 3-4 weeks later when the volunteer has recovered from the illness. All of the acute and a random sample of the convalescent nasopharyngeal specimens are inoculated into tissue cultures of primary monkey kidney (MK), Hep-2, HDCS WI28 and/or WI38, primary human embryonic kidney, semi-continuous human embryonic intestine and in addition recently into BS-C-1 cells also. Guinea pig erythrocytes are employed in hemadsorption tests in monkey kidney cell cultures for detection of myxoviruses while rat erythrocytes are employed similarly in BS-C-1 cell cultures for detection of OC43 related coronaviruses; rat red blood cells have been found recently to hemadsorb firmly to OC43 virus infected cell cultures (Project 72A).

The throat swab is tested for hemolytic streptococci (in our laboratory) and for hemophilus influenzae, streptococci and other bacteria, by the Clinical Center Clinical Pathology, Infections Laboratory Service (Dr. MacLowry). If B hemolytic streptococci are isolated they are grouped presently by the Infections Laboratory Service. Some of the nasal washings have also been tested for staphylococci with subsequent phage typing of recovered organisms (Dr. Dockstader).

In an attempt to find new agents, acute illness specimens which were negative for known viruses in the tissue cultures outlined above, are passaged three times in human embryonic tracheal organ cultures. The third passage harvest is clarified, concentrated, negatively stained with phosphotungstic acid, and examined by electron microscopy for the presence of coronavirus-like particles. In addition, the harvest from the 3rd passage is inoculated into the tissue cultures outlined above to determine if a cytopathogenic agent is recoverable. Recently, the additional technique of "immune electron microscopy" has been employed in an attempt to recover new agents, notably coronaviruses, from nasal washings which have failed to yield viral pathogens by tissue culture methods. A description of this technique is presented under project 72A "New Techniques for Recovery of Respiratory Tract Viruses".

Major Findings:

From September 9, 1965 to March 31, 1971 a total of 604 volunteers were admitted to the study. From 0 to 35 entered the study each month; some were in the study more than once. A viral agent was recovered from 242 (40%) of 600 specimens tested. Only one patient was B hemolytic group A streptococcus positive. One volunteer shed both a B hemolytic streptococcus (which was not grouped) and a rhinovirus-like agent as well; two viruses were recovered from one volunteer who shed both parainfluenza virus type 3 and an adenovirus. One-hundred fifty-eight (65%) of 242 virus isolations had tissue culture characteristics of rhinoviruses whereas 19 (8%) were identified as adenoviruses, 9 (4%) as herpes simplex, 9 (4%) influenza A-2, 7 (3%)

influenza B, 6 (2%) parainfluenza type 1, 6 (2%) parainfluenza type 2, 2 parainfluenza type 3, 1 parainfluenza type 4, 5 (2%) respiratory syncytial, 11 (5%) coronaviruses (6 229E, 2 OC43 and 3 other OC strain.) and 14 unclassified viruses.

The epidemiologic pattern of rhinovirus infections has been noteworthy as the frequency of such infections has decreased markedly in 5 of 6 winter seasons (December, January and February) following periods of relatively high prevalence during the preceding fall (September, October and November). For the 6 years combined rhinoviruses were recovered from 74 (36%) of 206 ill volunteers during the fall and from only 16 (9%) of 177 during the winter. This difference in rhinovirus isolation rate is statistically significant at the .0001 level. It was noteworthy that during the winter periods only 23 other viruses were recovered by conventional tissue culture techniques.

Attempts to recover the etiologic agents of respiratory illnesses observed during these fallow periods have been a major effort of this laboratory. These efforts have been fruitful with the use of two different culture techniques; human embryonic tracheal organ cultures and human embryonic intestine cell cultures. Details on the use of such cultures for detecting viruses during period of low rhinovirus prevalence have been described in previous annual reports. In brief, all 11 coronaviruses recovered in this study were isolated during the months of January, February and March; in addition, serologic studies have revealed that 18 (31%) of 59 patients studied from December, 1965 through February, 1966 developed 4-fold or greater CF antibody rises to OC38 and/or OC43 viruses, and 21 (24%) of 89 developed such rises to 229E virus during the 5 month period, December, 1966 through April, 1967. However, from December, 1967 through March, 1968 only 1 of 34 patients developed a coronavirus CF antibody rise and during the same period the following year 4 of 19 developed such rises in paired sera. Thus it appears that during some of the fallow periods coronaviruses are the predominant identifiable infecting organisms associated with non-influenzal adult respiratory disease.

Paired sera obtained from patients admitted to the study during the 4 year period from September, 1965 through August, 1969 were tested for serologic (CF) evidence of infection utilizing numerous respiratory agent antigens. 91 (20%) of 465 patients developed 4-fold or greater CF antibody rises in paired sera; 10 of the 91 had simultaneous rises to 2 agents of different antigenic groups making a total of 101 rises. Of the 101 rises, 11 were to the parainfluenza viruses, 3 to adenovirus, 5 to respiratory syncytial virus, 17 to influenza A virus, 9 to influenza B virus, 3 to Mycoplasma pneumoniae, 24 to coronaviruses OC38 and/or OC43 and/or MHV, and 29 to coronavirus 229E virus. It was noteworthy that more than one-half (53 of 101) of the CF antibody responses were to the coronavirus antigens. Further analysis of the data revealed that specimens from 461 patients were tested by both virus isolation and serologic techniques for evidence of infection during the 4 year period, September, 1965 through August, 1969 and evidence of infection by either virus isolation or serologic (CF) techniques or both was obtained in 247 (54%) of the 461 patients studied. In addition, one patient had a group A streptococcal infection.

Significance of the Program to the Institute:

These studies are aimed at evaluating the role of known viruses and bacteria as etiologic agents of adult respiratory diseases and at finding new agents which cause these illnesses. It was noteworthy that with the use of both virus isolation and serologic techniques over 50% of the illnesses studied among NIH personnel were found to be associated temporally with a viral agent. In addition, the presence of coronavirus infections during winter periods when non-influenzal respiratory illnesses were prevalent but virus isolation rates paradoxically low was noteworthy. It appears that with the use of new techniques such as organ cultures and experimental cell cultures new agents can be recovered during this previously fallow period. Elucidation of the etiology of the majority of upper respiratory illnesses during repeated respiratory disease seasons must be accomplished before attempts at prevention of such illnesses can be fruitful.

Proposed Course of Project:

These studies which attempt to find new agents associated with adult respiratory illnesses will receive major emphasis in this laboratory as recent efforts to recover such agents by using human embryonic organ cultures and experimental cell cultures have been highly rewarding. In addition, the role of bacteria alone or in association with viruses in the etiology of respiratory disease will be studied more extensively.

Honors and Awards: None

Publications:

Kapikian, A.Z.: Coxsackie and Echoviruses. In Beeson, P.B., and McDermott, W. (Eds.): *Cecil-Loeb Textbook of Medicine* (ed. 13), Philadelphia, Pa., W.B. Saunders Company, 1971, pp. 418-425.

Serial No. NIAID 70A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Laboratory Studies of Myxoviruses and Paramyxoviruses Associated with Respiratory Tract Disease

Previous Serial Number: Same

Principal Investigators: Dr. Elias G. Chalhub
Dr. Brian Murphy
Dr. Peter Wright
Dr. Robert M. Chanock
Miss Sandra Nusinoff

Contract Support:

Contract Number	Proj. Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	80/12			80/12
Professional:	44/12			44/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To produce conditional lethal temperature-sensitive (ts) mutants of myxoviruses and paramyxoviruses for possible use in live attenuated vaccines; (2) to study the genetic basis for attenuation shown by temperature sensitive mutants; (3) to define the relative importance of the various host defense mechanisms in resistance to myxovirus and paramyxovirus infection and disease.

Methods Employed:

A. Parainfluenza Viruses - In genetic studies, parainfluenza 1 or parainfluenza 3 virus is grown in African green monkey kidney (AGMK) culture and WI38 culture respectively and then purified by 3 successive terminal dilutions. Virus is then exposed to a chemical mutagen (5-fluorouracil) in the growth medium during viral replication. Clonal populations of the treated virus are then tested for capacity to grow at low or permissive temperature (30°-32°C) and at a high or restrictive temperature (38°-39°C). This evaluation is performed in 96 hole Falcon microplates which contain trypsinized AGMK or WI38 cells. A master plate is inoculated with a dilution of treated virus which will infect 10-20% of the wells. At this multiplicity of infection each infected well receives only one infectious unit in the inoculum. After 5-6 days of growth the master plate is replicated into two additional plates with sterilized steel replicators. The master plate is then frozen at -70°C. One of the replicated plates is placed at 32°C or the permissive temperature and the other at 39°C or restrictive temperature for 5-6 days. The replicated plates are then hemadsorbed with 0.1% guinea pig red blood cells. The viral populations in those wells which exhibit hemadsorption only at the permissive temperature represent the desired temperature-sensitive (ts) conditional lethal mutants. The appropriate wells are then identified in the frozen master plate and the provisional ts mutants are grown in AGMK or WI38 cell cultures to produce suspensions of virus which are then evaluated in tissue cultures, experimental animals and volunteers for temperature sensitivity and attenuation.

B. Influenza Virus - Influenza A virus suspensions were prepared in calf kidney tissue culture. In order to produce recombinant viruses, roller tubes of primary calf kidney (BK) were mixedly infected with wild type virus of the A2/HK/45/68 strain and a conditional lethal temperature sensitive (ts) mutant of the A2/Abbott/0389/65 strain. The A2/Abbott/65 mutant used was either ts-1 or ts-2, whose isolation and characterization was described in the previous annual report. Forty-eight hour harvest material from the mixed infection was incubated with antiserum specific for the A2/Abbott strain progeny (to select against parental ts A2/Abbott virus and recombinant wild type A2/Abbott progeny), and then inoculated into BK monolayers for plaque assay. Plaque progeny from this virus-antiserum mixture were picked and passed in BK roller tubes to increase their titer. These roller tube viral suspensions were then identified by the hemagglutination-inhibition technique and those bearing the Hong Kong hemagglutinin were further evaluated to determine whether they were recombinants bearing a ts defect. Identification was confirmed for prospective vaccine strains by plaque reduction neutralization tests in BK monolayers. Temperature sensitivity was determined by testing efficiency of plaque formation at 32°, 34°, 37°, 38° and 39°C. Clones of virus from mixed infection which appeared promising as recombinant vaccine strains were doubly plaque purified and then further characterized for basic genetic markers, using various experimental designs, in both primary and secondary passage BK monolayers. In addition to analysis of viral growth in tissue culture and in experimental animals, the growth characteristics of the strains were investigated in eggs. EID_{50} and extent of growth i.e., allantoic virus titer, were determined in regular and avian leucosis (RIF) free eggs.

C. RS Virus - Complementation analysis was performed by measuring the yield of mixed infection with 2 mutants at restrictive temperature and comparing this yield to that obtained with single infection. Heteroploid cell cultures infected with one of the RS ts mutants were fixed, sectioned and examined by electron microscopy to determine the morphogenesis of defective virus at restrictive temperature.

D. Immunologic Factors in Host Defense and Resolution of Infection - The pattern of infection of hamsters given RS virus or rabbits given type 3 parainfluenza virus was studied in normal animals and in animals whose host defenses had been compromised or ablated by X-irradiation or cyclophosphamide.

Major Findings:

A. Conditional Lethal Temperature Sensitive (ts) Mutants of Parainfluenza Viruses - The type 1 parainfluenza virus was isolated in AGMK tissue culture from a child with an illness of the upper respiratory tract and was passaged twice in AGMK at 35°C. This virus was cloned 3 times in AGMK roller tubes at 37°C and then grown in the presence of 2.5 mm 5-fluorouracil, a concentration of mutagen which produced a 100-fold suppression of virus yield at 48 hours. Mutagenized virus was then placed in Falcon microplates as outlined above and characterized for temperature sensitivity. Four potential mutants have been recovered thus far. These mutants were numbered sequentially and stock suspensions prepared for further characterization of the ts defects.

The type 3 parainfluenza virus was isolated in WI38 tissue culture from an adult with an upper respiratory tract illness. It was passaged 6 times in WI38 cells at 37°C and was designated as the parent virus. The virus has been cloned 3 times in WI38 cells and is currently being treated with 5-fluorouracil.

B. Influenza A Virus -

(1) Recombination between strains of influenza--transfer of temperature sensitive defect: We were unable to evaluate the level of attenuation of conditional lethal temperature sensitive (ts) mutants of the A2/Abbott/65 strain because all of our volunteers possessed moderate to high titers of neutralizing antibody for this virus. This led us to produce ts mutants from a more recent A2/HK/45/68 strain of influenza, for which a significant segment of the population remains seronegative. Influenza virus characteristically exhibits very high frequencies of "recombination" when two similar strains are grown in mixed infection. To obtain ts mutants of influenza A2/Hong Kong, wild-type virus of this strain was grown in primary calf kidney cultures which were simultaneously infected with either the ts-1 or ts-2 mutant of the influenza A2/1965 virus. The progeny from these mixed infections were exposed to various selection procedures as described above to obtain desired influenza

recombinants which possessed the A2/HK/68 hemagglutinin and the ts defect(s) of the parental A2/1965 strain. Seventy-two plaque progeny from the A2/Hong Kong/68 X A2/65 ts-2 cross were analyzed. Fifty-four possessed the desired Hong Kong hemagglutinin and 10 of these were also temperature sensitive. Similarly, in the A2/HK/68 X A2/65 ts-1 cross, of 60 viable plaques originally screened, 57 possessed the Hong Kong hemagglutinin, of which 13 also were temperature sensitive.

Characterization of these recombinant virus strains not only revealed that it was possible to transfer temperature sensitive defects from one strain of influenza A to another through the relatively simple experimental device of recombination, but also provided additional information on the general nature of these defects and their localization in the influenza genome. Insight into the nature of the ts lesions came from a detailed study of temperature sensitivity and its segregation during recombination. Detailed temperature sensitivity determinations were made by evaluating the efficiency of plaque formation of the parental and recombinant strains at permissive temperatures of 32°C, 34°C and restrictive temperatures (depending on the particular strain being used) of 37°, 38°, and 39°C. When it was necessary to maintain critical control, constant temperature circulating water baths were used. All recombinant virus strains from the A2/Hong Kong/68 X A2/65 ts-2 cross were like their ts-2 parent in exhibiting essentially uninhibited growth in the range 32°-37°C, slight inhibition (approximately 10-fold reduction in plaque formation) at 38°C, and no detectable growth at 39°C. Analysis of the temperature sensitivity patterns of the recombinant progeny from the A2/Hong Kong/68 X A2/65 ts-1 cross revealed a somewhat different situation. While some of the recombinant Hong Kong ts-1 strains exhibited a temperature range similar to the parental ts-1 (of the A2/65 strain), growing essentially unhibited at 32° and 34°C but showing no appreciable growth at 37°, 38°, or 39°C, another group of the Hong Kong ts-1 recombinants exhibited patterns of growth similar to that of the ts-2 strains, in that they grew to equivalent titers at 32° and 37°C, were slightly depressed in titer at 38°C, and showed no growth at 39°C. Of 13 recombinant A2/HK/68 ts-1 strains isolated, four showed the parental pattern of temperature sensitivity (with shut-off temperatures of 37°C), while the other 9 strains shut off at 39°C in a manner similar to ts-2.

The segregation of two classes of ts-1 Hong Kong recombinants provides information concerning the nature of the temperature sensitive lesions of the original ts-1 mutant. It was shown by multiple passage in calf kidney tissue culture of both the parental A2/65 ts mutants and the recombinant ts mutants that the lesions causing temperature sensitivity are stable genetic defects. Reversion frequencies were calculated to be less than 10^{-5}. This suggested that the temperature sensitivity of the original ts-1 was not due to a single mutation but a constellation of defects. With the two parental ts mutants, one with a shut-off temperature of 37°C and the other shutting off at 39°C, two assumptions for the genetic basis of temperature sensitivity are possible. One could assume either that the temperature of shut off was due to degree of genetic damage (i.e., the absolute number of lesions, the mutant shutting off at 37°C being quantitatively more damaged) or that specific lesions or clusters of lesions caused the differing shut-off characteristics. The isolation of two classes of ts recombinants from the original parental ts-1 (37°C shut-off temperature) indicated that there was indeed more than one lesion present in the genome of that virus. However, genetic studies were needed to determine whether the 39°C ts-1 recombinant possessed only one lesion while the 37°C ts-1 recombinants possessed qualitatively different, non-overlapping lesions. Analysis of the lesions in the two different classes of recombinant HK ts-1 mutants was accomplished by use of the plate complementation technique. This consists of doubly infecting BK monolayers with the two mutants to be studied, and observing their ability (or lack thereof) to synergistically produce plaques under agar at a temperature restrictive for the growth of either one of the mutants. It was found that if cells were doubly infected with ts-1 recombinant strains of the same type, i.e., 2 strains which shut off at 37°C or two strains which shut off at 39°C, plaque formation was not detected at the restrictive temperature, while each grew to subsequential titer at the permissive temperature. If, however, the cells were doubly infected with one recombinant exhibiting a 37°C shut off and the other shutting off at 39°C, plaques did form at the restrictive temperature. The two classes of ts-1 recombinants presumably were each defective in a different part of the genome and could supply together at the restrictive temperature all components required for virus replication. It was also shown by the plate complementation technique that neither the Hong Kong ts-1 clones which shut off at 37°C or those which shut off at 39°C were complemented at the restrictive temperature by the parental A2/65 ts-1 mutant. Both classes did, however, produce plaques when mixed with the A2/65 ts-2 mutant at the restrictive temperature, as did their parent ts-1 mutant. The ability of the 39°C shut off ts-1 recombinant to complement the 39°C shut off ts-2 recombinant indicated the existence in these mutants of 2 different temperature sensitive lesions which resulted in the same phenotype, i.e., 39°C shut off. Each of these mutants have defects in different biochemical functions, while from the gross biological criteria of ability to form plaques at a specified temperature or behavior in animals they are indistinguishable. In summary it appears that the original ts-1 mutant of influenza A2/1965 has at least two lesions (or clusters of lesions)--one defect prevents plaque formation at 37°C while the other imposes a somewhat higher temperature sensitive limitation of 39°C. The original ts-2 mutant of influenza A2/1965 possesses a lesion (or lesions), distinct from those of the ts-1 mutant, which imposes a temperature limitation of 39°C. Recombinants of the ts-1 mutant with Hong Kong influenza A appear to have one of the ts-1 lesions but not both.

(2) Evaluation of recombinant temperature sensitive mutants in experimental animals - After laboratory characterization and identification of the recombinant A2/HK/68 ts mutants, as discussed above, virus suspensions were prepared for study of the mutants in laboratory animals prior to evaluation in human volunteers. Both hamsters and mice were inoculated intranasally with parental wild type viruses, parental ts mutants, and recombinant ts mutants, and the growth patterns of these strains compared. The animals were sacrificed at 1, 2, 3, and 4 days post-inoculation and the pulmonary virus titer in each animal determined by titration of 10% weight/volume lung suspension. The wild-type parental strains, both A2/Hong Kong/68 and A2/Abbott/65, showed maximum growth to a titer of $>10^5$ $TCID_{50}$ per 0.2 ml lung suspension on day 1 post-inoculation. Virus titers gradually declined thereafter and virus was cleared by day 4 or 5. With the parental A2/Abbott/65 ts-2 mutant (39°C shut-off temperature) virus titers in the lungs rose more gradually to a peak on day 2 and then declined, the virus clearing at about the same time as the wild type. The peak pulmonary virus titer of ts-2 was approximately 100-fold less than that seen with wild type virus. The parental A2/Abbott/65 ts-1 mutant (37°C shut-off temperature) was limited in pulmonary growth to an even greater degree. Virus was detected in the lungs of the animals only on day 2, and the geometric mean titer was less than 10^1 $TCID_{50}$/0.2 ml lung suspension.

Pulmonary virus titers of animals infected with the recombinant ts mutants generally followed the patterns exhibited by their parental strains. There were, however, several exceptions to the general pattern. All the ts-2 (39°C shut-off) recombinant mutants showed delayed growth patterns, and none reached a titer equivalent to that of the wild type strains. However, some of the recombinants did grow better than the parental A2/Abbott/65 ts-2. As indicated previously, the ts-1 recombinants fell into two genetically distinct classes, one of which possessed a shut-off temperature like that of its ts-1 A2 parent (37°C) and another which shut off at 39°C. These two classes of recombinants also had different growth properties in animals. The low shut-off ts-1 recombinants behaved like their A2/65 parent mutant in exhibiting minimal growth in the lungs. Those ts-1 recombinants which shut off at 39°C, however, behaved much like the ts-2 strains which shut off at 39°C, growing to intermediate titers in the host, with a delayed growth cycle.

It was shown that inoculation of animals with either ts-1 or ts-2 recombinants stimulated the production of serum antibody and induced resistance to challenge with wild type Hong Kong influenza A virus. Moreover, titer of serum antibody titer was related to the degree of resistance to challenge shown by the experimental host. Inoculation with wild type virus stimulated the highest antibody titers while ts-2 stimulated titers which were slightly lower and ts-1 stimulated somewhat lower levels of antibody. The resistance induced by the ts mutants was specific since it did not provide protection against influenza B virus.

The general laboratory experience with these strains and their behavior in animals, as well as the studies done with these strains in human volunteers (see NIAID 72B), indicates that temperature-sensitive mutants do appear to have definite potential as live virus vaccine strains. They can indeed be shown to grow in the nasopharynx and to a limited degree in the lungs (depending upon the extent of their restrictive temperature) and also to stimulate the production of protective antibody. It is also clear, however, that the strains presently being evaluated in this laboratory are not the ideal strains for use in a vaccine. The 39°C shut-off temperature appears to allow viral replication to occur to a degree greater than is desirable, whereas mutants with a 37°C shut-off defect are not capable of sufficient replication to stimulate a high level of resistance.

C. Conditional Lethal Temperature-Sensitive (ts) Mutants of RS Virus - A new RS ts mutant, ts-7, was isolated by Louis Potash, Flow Laboratories, and made available to us for genetic studies. This mutant was derived from virus treated with ethyl methyl sulfonate (EMS). The other mutants (ts 1-6) recovered in this laboratory were induced by growing virus in the presence of a base analogue,5-fluorouracil or 5-fluorouridine.

The 7 ts mutants of RS virus were analyzed for evidence of complementation and pseudo-recombination (a manifestation of complementing heterozygotes). Instead of forced air incubators, constant temperature water baths (temperature ±0.05°C) were used for cultures incubated at the restrictive temperature. This more precise and reliable temperature control was apparently responsible for a marked decrease in the frequency of pseudo-recombination produced by complementing heterozygotes. Complementation analysis indicated that the new mutants recovered in our laboratory, ts-5 and ts-6, belong to a complementation group previously shown to contain ts-1, ts-3 and ts-4. Ts-7 was found to be a representative of a separate complementation group. At present we have evidence for the existence of 3 RS complementation groups; group 1, ts-1, 3, 4, 5 and 6; group 2, ts-2, and group 3, ts-7. Each of the mutants was studied for its stability at 39°C for 2 hours. Except for ts-2, the atypical plaque mutant, each of the mutants was more heat labile than wild type virus suggesting that the ts defects in groups 1 and 3 affected structural proteins.

With the aid of the electron microscope we have attempted to define the stage of replication at which the ts mutants were blocked by comparing the morphology of virus infected HeLa cells incubated at permissive or restrictive temperature. At the restrictive temperature 2 of the mutants belonging to group 1, ts-1 and ts-4, have an apparent block in release of virus by budding from the cell membrane. Large cytoplasmic factories of what appears to be viral ribonucleoprotein develop at restrictive temperature, but maturation of virus at the cell surface occurs with considerably reduced frequency. The ts-2 mutant does not appear to induce formation of multinucleated cells, even at permissive temperature.

This finding confirms observations made by light microscopy. During the past year we have developed an in situ embedding and sectioning technique which enables us to analyze a single syncytia without distortion caused by scraping and pelleting the cell sheet.

D. Evaluation of the Role of Immunologic Factors in Host Defense to RS Virus Infection in Hamsters - Evidence that immunological factors are of importance in the pathogenesis of RS virus infection in humans comes from 2 sources: (1) the virus produces its most severe disease manifestations in infants in the first 3 months of life when maternally transmitted antibody is present; and (2) inactivated parenterally administered vaccine induces a state of altered reactivity so that vaccinees develop an exaggerated illness when infected with RS virus. It was felt that an understanding of the relationship between enhanced disease and presence of circulating antibody might be derived from an investigation of immunologic factors in the pathogenesis of RS virus in hamsters, the only convenient laboratory animal which sustains replication of RS virus throughout the entire respiratory tract. First, the effect of X-irradiation upon RS virus infection was investigated. The LD_{50} for 4 week old hamsters was found to be 700 R. Based upon this finding 500 R, a non-lethal dose, was used to ablat or seriously impair the immunological responsiveness of the hamster. Four week old hamsters were irradiated (500 R) 2 days before being given 2 X 10^5 pfu of RS virus intranasally. At regular intervals the lungs, nasal turbinates and blood were assayed for content of virus, antibody and interferon. In the irradiated-infected animal virus persisted in the lungs and nasal turbinates until day 14, whereas unirradiated hamsters were free of virus by 4-5 days. In association with the delayed clearance of virus in the X-irradiated animal we observed a delay in the appearance of serum antibody; antibody was not detected until day 10, whereas unirradiated animals developed antibody by day 6. Interferon was not detected in the serum or lungs of irradiated or unirradiated infected hamsters. Whether the delay in antibody development produced by X-irradiation was responsible for the delay in clearance of virus remains to be determined.

E. Evaluation of Host Defense Factors in Recovery of Rabbits From Infection with Parainfluenza Type 3 Virus - The multiple factors contributing to recovery from primary or secondary respiratory viral infection remain incompletely defined. Infection of rabbits with type 3 parainfluenza virus was chosen as a model system for study of the relative contributions of the various host defense factors in recovery from first and second exposure to a virus. This was done for the following reasons: (1) the immunologic and interferon systems are well defined and easily quantitated in the rabbit; (2) the size of the animal permits easy experimental manipulation; and (3) the interstitial pneumonia produced in the rabbit is similar to that seen during natural infection of man. First, the role of the immunologic response in resolution of primary viral infection was studied. After the LD_{50} of cyclophosphomide for 600-700 gm New Zealand white rabbits was determined to be 175 mg/kg, a non-lethal dose of 100 mg/kg was administered intraperitoneally one day after intranasal inoculation of 1 x 10^5 pfu of type 3 virus. This dose of cyclophosphamide did not cause a depression in the peripheral white blood cell count. In cyclophosphamide-treated rabbits virus persisted at high titer in the lungs for a significantly longer interval compared to the non-drug treated infected animals. This prolonged pulmonary infection was accompanied by a delay in the appearance of serum antibody. Interferon was detected only in the lungs of the cyclophosphamide-treated animals. Microscopic examination of the lungs for pathology demonstrated a more severe interstitial pneumonia in the cyclophosphamide-treated infected animals. These results demonstrated that first exposure to type 3 virus in an immunosuppressed rabbit resulted in a more extensive infection as manifested by prolonged viral replication and more extensive pneumonia. The mechanism for this enhancement remains to be defined, but it is interesting that higher titers of interferon were detected in the more severely infected animals, suggesting that immunological factors play a major role in recovery from primary type 3 virus infection.

Significance of the Program to the Institute:

Since our initial recovery of parainfluenza viruses from man 12-14 years ago, clinical and epidemiological studies have established these agents as important pathogens of the respiratory tract. During primary infection with parainfluenza 1, the lower respiratory tract is often involved, and illness may be quite severe. Type 3 parainfluenza virus produces a spectrum of clinical illnesses in children including rhinitis, pharyngitis bronchitis, croup, bronchiolitis, and bronchopneumonia. Thus, it is clear that immunoprophylaxis is needed to thwart the impact of these viruses in infants and young children. Inactivated parainfluenza virus vaccines have not provided significant resistance to respiratory illness caused by these viruses in infants and children. For this reason we have turned our efforts toward the development of live attenuated vaccines which will stimulate protective local immunity in the respiratory tract without causing disease.

Similarly, influenza A virus continues to be an important cause of respiratory tract disease morbidity and mortality although relatively effective protection can be provided by administration of a sufficient quantity of inactivated antigen by the parenteral route. In our view local respiratory tract immunity can be induced more efficiently by infection with an attenuated virus. In addition to this advantage an attenuated vaccine would probably be more readily accepted by the public and thus have a greater impact in prevention of disease. These theoretical advantages are sufficiently attractive to stimulate us to continue our efforts to develop mutants of influenza virus with the desired degree of attenuation and immunogenic activity. The current findings are of significance to the Institute's program of influenza immunoprophylaxis in that partial success has been achieved. It is now clear that ts mutation leads to attenuation of influenza virus. Attenuation appears to be a phenotypic manifestation of the ts lesion(s) since transfer of the lesion(s) by recombination leads to an associated transfer of attenuation to the recombinant virus. This also means that live vaccines can be updated at the time new antigenic variants emerge by recombination and transfer of the ts lesion(s) to the new virus. The success of this approach was demonstrated this year when we transferred ts lesions (and the associated attenuation) of an influenza A2 1965 virus to the influenza A Hong Kong 1968 virus. Of course this method of transferring attenuation to new influenza viruses requires that the ts lesions not affect that portion of the genome which codes for the hemagglutinin. Since we were fortunate in inducing ts mutations in the non-hemagglutinin portion of the genome the transfer of ts lesions and attenuation to a new influenza virus could be achieved.

In addition to demonstrating the feasibility of the ts approach to attenuation of influenza virus several interesting observations concerning the nature of the mutations were made. In tests with the original 1965 ts mutants and those performed with the Hong Kong 1968 ts recombinants there was a strong association between degree of temperature defectiveness in vitro and the extent of limitation of growth in vivo. This relationship, which was not surprising, should prove useful for the purpose of predicting level of attenuation of new mutants or recombinants in future studies. Finally, we were able to identify 3 discrete ts lesions in the 2 mutants which were studied most extensively. One of the mutants, ts-1, contained 2 separate lesions--one lesion imposed a temperature limitation of 37°C, while the other produced a limitation of growth at 39°C. The second mutant, ts-2, had a distinct lesion which also imposed a limitation of growth at 39°C. This information should prove useful in future studies in identifying similar or distinct lesions present in other mutants or recombinants. In this context it should be mentioned that the existence of 2 distinct lesions (or clusters of lesions) in the ts-1 mutant did not become evident until recombination with the Hong Kong virus yielded 2 classes of recombinants which complemented each other.

Recognition of 3 complementation groups among the 7 ts mutants of RS virus represents an advance in our understanding of the relation of these mutants to each other. It now appears that defects in at least 3 distinct cistrons have been identified. Members of a complementation group share a defect in at least one cistron and this cistron is not affected in mutants belonging to the other 2 complementation groups. This information is of importance to the program of vaccine development in that we can now identify mutants which share a common defect. This simplifies the choice of mutants to be evaluated in man, since initial trials should include the testing of a representative from each complementation group rather than a series of mutants with the same defect.

Studies of the role of various host defense mechanisms in recovery from respiratory tract infection are clearly relevant to the Institute's program for control of this type of disease. A better understanding of the relative importance of the different defense systems should prove to be of inestimable value in planning the most effective means for control of respiratory tract illness.

Proposed Course of Study:

Study of the ts mutants of the parainfluenza viruses will continue in an effort to determine their potential for use in a live virus vaccine. Evaluation of the mutants for degree of defectiveness and attenuation will be conducted first in experimental animals and then in young adult men and subsequently in young children.

Additional genetic manipulation of the ts strains presently available will be attempted with the aim of selecting new ts recombinants with the desired properties. Also, the A2/HK/68 strain of influenza will be mutagenized with 5-fluorouracil, in a manner similar to that used to generate the original A2/65 mutants (see previous annual report 70A Methods) and this mutagenized virus suspension will be examined for new ts mutants with the desired properties. By passage of the A2/HK/68 ts-1 volunteer pool (previously administered to volunteers at the Lorton Reformatory) in avian leukosis (RIF) free eggs and in the presence of minimal doses of hydrocortisone it has recently been possible to produce a suspension of the HK ts-1 recombinant with a titer of 10^7 pfu/ml compared to a titer of slightly more than 10^4 pfu/ml when grown in calf kidney tissue culture. This high titered egg material is being safety-tested and will be evaluated in volunteers to determine if a larger dose of a mutant which shuts off at 37°C can produce a silent infection which provides protection against subsequent wild type challenge.

Additional ts mutants of RS virus will be sought and then compared by complementation analysis in an effort to obtain mutants with defects distinct from those already available. Attempts to define the stage in replication of RS virus at which different mutants are blocked will continue.

In order to further elucidate the role of immunologic factors in the pathogenesis of RS virus in the hamster the following studies are planned: (1) the role played by delayed hypersensitivity will be studied by employing rabbit-anti-hamster thymocyte sera to impair cell mediated immunity; (2) hamster anti-RS serum will be given to irradiated animals to determine if serum antibody plays a role in resolution of infection or immunologic injury.

Studies are planned to investigate in greater depth the role of interferon, the alveolar macrophage, delayed hypersensitivity, and local and systemic antibody in resolution of primary infection and re-infection with type 3 parainfluenza virus. We intend to further define the contribution of these various factors in resistance to infection. This information should prove helpful in designing a vaccine that will cause maximal stimulation of the relevant factors that determine resistance to this viral infection.

Honors and Awards: None

Publications:

Mills, J., and Chanock, R.M.: Temperature-sensitive mutants of influenza virus I. Behavior in tissue culture and in experimental animals. J. Infect. Dis. 123: 145-157, 1971.

Potash, L., Lees, R.S., Greenberger, J.L., Hoyrup, A., Denney, L.D., and Chanock, R.M.: A mutant of parainfluenza type 1 virus with decreased growth capacity at 38° and 39°C. J. Infect. Dis. 121: 640-647, 1970.

Wright, P.F., Woodend, W.G., and Chanock, R.M.: Ts mutants of respiratory syncytial virus: In vivo studies in hamsters. J. Infect. Dis. 122: 501-512, 1970.

Serial No. NIAID 70B
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Classification and Characterization of Rhinovirus Serotypes of Human Origin

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: 8 Collaborating U.S. Laboratories and 2 WHO International Reference Centers (Common Cold Research Unit, Salisbury, Wilts, England, and Baylor University, Houston, Texas)

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Dir.	Annual Funding Level
PH 43-65-562	Dr. Kapikian	Children's Hosp. Columbus, Ohio	Dr. Hamparian	$142,960
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs.	Dr. Gilden	135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	7/12			7/12
Professional:	3/12			3/12
Other:	4/12			4/12

Project Description:

Objectives: (1) To classify the numerous rhinovirus serotypes in order to develop a suitable nomenclature system; (2) to characterize rhinovirus serotypes antigenically; (3) to elucidate the interrelationships between different rhinovirus serotypes.

Methods Employed and Major Findings:

Rhinovirus Collaborative Program - The first phase of this collaborative program which was designed to characterize and enumerate the number of distinct rhinovirus serotypes and which was described in detail in a previous annual report instituted a new numbering system for rhinoviruses based upon extensive cross-testing of a large number of strains. This numbering system which was approved by the Directors of the World Health Organization Respiratory and Enterovirus Reference Centers enumerated 55 distinct serotypes from 1-55 with one subtype. The second phase of the program which was recently completed and reported in detail in last year's annual report, added 34 additional serotypes thereby extending the numbering system to include rhinoviruses 56 through 89. A third phase of the program including certain second phase viruses which demonstrated one-way or unresolved relationships, is in progress. The third phase will also include those prototype candidate viruses which are found to be distinct from previously numbered serotypes.

Characterization of Rhinoviruses Obtained from Epidemiologic Studies - 126 viruses which were recovered from patients enrolled in the LID, NIH Common Cold Study (Project 69C) and which produced rhinovirus-like CPE in tissue cultures have been tested for acid sensitivity and all but 2 were found to be acid sensitive; in addition, 104 of these acid sensitive viruses were tested for chloroform sensitivity and all but one were chloroform resistant. The acid labile, chloroform resistant viruses were classified as rhinoviruses on the basis of these biochemical studies. With the completion of the second phase of the collaborative program and the availability of small amounts of antisera for all numbered serotypes, attempts are being made on a small scale to type some of these rhinovirus isolates. Such a limited study will not only provide valuable information on the approximate number of uncharacterized serotypes, but also yield epidemiologic information on the prevalence of different serotypes in this community. Thus far, 6 acid labile, chloroform resistant viruses have been tested against 90 antisera representing the 89 serotypes and the one subtype; 4 were typed (two as rhinovirus (R) 2, one as R19 and one as R42) and two were not neutralized by any of the 90 sera; the 2 untyped viruses represent either one or 2 new serotypes which will be submitted to the third phase of the collaborative program after further characterization is completed. Acid labile viruses obtained from studies at Junior Village are described in Project 66A.

Immune Electron Microscopy of Rhinoviruses - While a guest worker in 1970 at the Royal Postgraduate Medical School, London, England in Professor Anthony P. Waterson's Department of Virology, one of the studies which was carried out in collaboration with Mrs. June Almeida involved the electron microscopy of rhinoviruses.

These viruses have proven difficult to visualize in simple tissue culture preparations by conventional negative staining techniques. This is probably related to difficultites in obtaining high titered preparations but also possibly to the inherent instability of the rhinovirus particle. However, excellent micrographs of rhinoviruses were obtained employing the slight modification of the negative staining technique which had been previously successfully applied to rubella virus, (J.M. Best, J.E. Banatvala, J.D. Almeida and A.P. Waterson). This modification comprised the addition of specific antiserum to the virus-containing preparation so that the particles gathered in the form of aggregates rather than individual units. Specimens in which the virus suspension had been mixed with saline alone displayed randomly scattered particles but since rhinoviruses do not display a very distinctive morphology by negative staining there may have been more particles than were recognized. In order to avoid confusion with small protein aggregates only those particles that displayed distinctive virus form were considered to be rhinoviruses. Reaction with heterotypic antiserum resulted in mostly randomly scattered virus particles but there were also occasional aggregates. Reaction of 3 rhinovirus serotypes (one grown in HeLa spinner cultures--supplied by Dr. R. Buscho, one in L-132 cells, and clarified--supplied by Dr. J. Stott, and one in WI38 cells) with homotypic sera resulted in the appearance of both large and small immune aggregates. The particles were no longer randomly distributed but were present as groups or aggregates which stood out clearly from the surrounding cellular debris. Virus aggregates could even be visualized after ultracentrifugation of virus-serum mixtures for only 1/2 hour at 12,000 rpm (The usual procedure was to ultracentrifuge the virus-serum mixtures for 90 minutes at 18,000 rpm). The amount of antibody present influenced the definition of the virus particles. As might be expected, the optimal concentration for the antibody was in the region of equivalence; under these conditions there was maximal clumping of particles but minimal amounts of antibody.

Examination of the virus clumps revealed that there was a reasonably distinct division into aggregates containing complete virus particles, as judged by their white appearance in the negative stain, and incomplete particles distinguished by their ring appearance. The division was not completely clear-cut, however, and occasional mixed aggregates were found. The aggregates most clearly seen were those composed of whole particles which were approximately 200 Angstroms in diameter and which displayed a smooth outer surface on which subunits could not be resolved. The different composition of the aggregates suggests that rhinoviruses may display distinct antigenic properties depending on whether they are "full"or "empty" as previously described for poliovirus (K. Hummeler, T.F. Anderson and R.A. Brown). Another feature was the tendency of the rhinoviruses to show an appearance of disintegration within the immune complexes. This tendency was associated with already partially degraded incomplete particles. There are two possible explanations for this phenomenon: (1) that the virus itself is physically extremely unstable and that spontaneously degraded particles are actually preserved by the stabilizing effect of adsorbed antibody and the particles are then contained within what could well be a protective complex; or (2) it may be that the presence of antibody on rhinovirus capsids may itself lead to degradation of the rhinoviruses.

Significance of Program to Institute:

Rhinoviruses are the most important known group of viruses to be associated with acute upper respiratory tract illnesses in adults. However, the rapidly increasing number of serotypes and the absence of an acceptable numbering system made interpretation of epidemiological data difficult and also prevented satisfactory epidemiological investigations of rhinovirus infections. The numbering system will permit such studies to be undertaken satisfactorily and such studies will be important in evaluating the need for rhinovirus vaccines.

Proposed Course of Project:

It is anticipated that the third phase of the collaborative program will include significantly fewer viruses than in either the first or second phases. In addition, studies on the different antigenic components of individual rhinovirus serotypes will be expanded using the technique of immune electron microscopy; the effects on viral aggregation by different immunoglobulin groups, such as IgA will also be investigated.

Honors and Awards:

Publications:

A Collaborative Report: Rhinoviruses-Extension of the Numbering System. Virology 43: 524-526, 1971. Members of the group: A.Z. Kapikian (chairman), R.M. Conant, V.V. Hamparian, R.M. Chanock, E.C. Dick, J.M. Gwaltney, Jr., D. Hamre, W.S. Jordan, Jr., G.E. Kenny, E.H. Lennette, J.L. Melnick, W.J. Mogabgab, C.A. Phillips, J.H. Schieble, E.J. Stott, D.A.J. Tyrrell.

Kapikian, A.Z., Chapter on Rhinoviruses in Strains of Human Viruses (In press).

Serial No. NIAID 70C
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Mycoplasmas

Previous Serial Number: Same

Principal Investigators: Dr. Helmut Brunner
Dr. Shmuel Razin
Dr. Robert Purcell
Dr. Robert Chanock

Other Investigators: Dr. Benjamin Prescott (LM)
Mr. Anthony Kalica (LID)

Cooperating Units: None

Contract Support: None

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	44/12		12/12	56/12
Professional:	20/12		12/12	32/12
Other:	24/12			24/12

Project Description:

Objectives: (1) To study the biological and biophysical properties of mycoplasmas, with particular emphasis on *Mycoplasma pneumoniae*, the etiologic agent of cold agglutinin positive pneumonia, and to determine the relationship of those properties to virulence; (2) to develop more sensitive and specific serologic techniques for the measurement of antibody to mycoplasmas; (3) to employ these techniques for the seroepidemiologic study of diseases of obscure etiology; (4) to test the possibility of using hybrid reaggregates of the serologically-active lipids of *M. pneumoniae* and membrane proteins of *M. laidlawii* as an immunizing agent in the hamster model; (5) to study the infectivity stability and protective capacity of ts mutants of *M. pneumoniae* (strain Pl 1428) in the hamster animal model.

Methods Employed:

(1) Mycoplasmas were grown on agar plates or in broth suspension. *M. pneumoniae* was also grown in Povitsky bottles under conditions in which the colonies became attached to the glass surface.

(2) The complement mediated killing reaction first described by Gale and Kenny for M. pneumoniae was made more sensitive by incubating organisms in the presence of serum for 60 minutes at 4°C prior to the addition of fresh guinea pig serum which served as the source of complement. In the modified killing or mycoplasmacidal test the decrease in viability of M. pneumoniae produced by various concentrations of antiserum was determined in the presence of a constant amount of fresh guinea pig serum, and the highest dilution of test serum which produced a 90% killing effect was estimated after logit transformation using a computer program.

(3) A radioimmunoprecipitation method which employed small quantities of reagents (0.025 ml) and microtiter equipment was developed. The organisms were grown in 2 liter Povitsky bottles in the presence of C^{14}-oleic acid and C^{14}-palmitic acid. The cell sheet adhering to the glass surface was washed 3-4 times after decantation of the medium. These organisms were filtered through a 450 nm millipore filter and diluted to an activity of 500-1000 cpm per 0.025 ml. Radiolabelled antigen was incubated with different serum concentrations for 60 minutes at 37°C and then overnight at 4°C. The antigen-antibody complexes were precipitated by anti-human globulin produced in germ-free guinea pigs. After centrifugation of the precipitates at 1000 rpm for 10 minutes, the radioactivity remaining in the supernatant was determined. From this determination the highest serum dilution which gave a 25% binding of antigen was calculated and considered to be the titer.

(4) Syrian hamsters were inoculated intranasally with either temperature sensitive (ts) or wild type M. pneumoniae. Response of the animals to infection was measured by growth of the organism in the lungs and nasal turbinates, appearance of MI, CF and mycoplasmacidal antibody in the serum, and lung histopathology. Six weeks after initial infection, the animals were challenged intranasally with a virulent wild type M. pneumoniae strain and the response to challenge studied as above.

(5) Syrian hamsters were immunized with reaggregates of M. pneumoniae lipids and M. laidlawii membrane protein. Six weeks later the animals were challenged with wild type M. pneumoniae and the infectivity determined as described in 4.

Major Findings:

A. More Sensitive Serologic Techniques for Measurement of Antibody to M. Pneumoniae - At present, metabolic inhibition (MI) and complement fixation (CF) are the most widely used methods for serodiagnosis of M. pneumoniae infections, but methods with increased sensitivity for detection of antibody are needed to illuminate certain unexplained aspects of M. pneumoniae infection. For example, there is the unusual age distribution of disease which occurs with greatest incidence in the 5-15 year age group. Then, there is the recent experience at Children's Hospital of D.C. in which an unexpectedly high frequency of shedding of M. pneumoniae was observed in young children who were not ill (Canchola, J., Kim, H.W., Brandt, C.D., Parrott, R.H., and Chanock R.M., et al., to be published). Taken together these findings suggest that M. pneumoniae disease might result from re-infection of individuals previously sensitized to the organism. In order to explore this possibility highly sensitive methods for assay of M. pneumoniae antibody are required. We were also interested whether or not local antibody in respiratory tract secretions might play an important role in resistance to disease. Previous attempts to detect antibody in local secretions had failed, but this failure may have resulted from an insensitivity of the immunologic methods used.

Both mycoplasmacidal and radioimmunoprecipitation (RIP) tests were used to study paired sera of volunteers collected prior to and 28 days after challenge with M. pneumoniae, strain PI 898. The mycoplasmacidal and RIP techniques were considerably more sensitive for detection of antibody to M. pneumoniae than the previously employed methods. Furthermore, the mycoplasmacidal and RIP methods measured large increments in antibody during convalescence from experimental infection. There was good agreement when the results of the two methods were compared with one another. The radioimmunoprecipitation method appeared to be slightly more sensitive than the mycoplasmacidal technique. It is quite clear that these tests are considerably more sensitive than the metabolic inhibition test. In addition, antibody was measurable by the mycoplasmacidal test when indirect immunofluorescence, metabolic inhibition and complement fixation (even employing highly specific membrane glycolipids as antigen) failed to detect antibody. A significantly higher mycoplasmacidal antibody titer ($p < 0.05$) was observed in pre-challenge sera of volunteers who did not become ill as compared to those who developed illness, indicating that this antibody was related to resistance to M. pneumoniae disease.

In a preliminary study infants 7 to 12 months of age, without respiratory tract disease, were found to have a significantly lower mean mycoplasmacidal antibody titer than children one to two years of age who were also free of respiratory disease. Similarly the infants had a significantly lower titer than a group of normal adults. The relatively high frequency and titer of antibody in sera of one to two year old children gives some preliminary support to the hypothesis that disease might be favored by prior sensitization induced by infection at an early age.

B. Mechanism of Antibody Mediated Killing of M. Pneumoniae - The cell killing reaction produced by M. pneumoniae antiserum was shown to be mediated by antibody specific for the glycolipid hapten of the organism. In the presence of complement the organism was killed by a rabbit serum which contained antibody for the glycolipid hapten but not for the other constituents of M. pneumoniae. Complement was essential for killing of the organism. Apparently, viability was destroyed by lysis of the mycoplasmal membrane effected by antibody and complement operating in concert. This reaction was visualized by negative staining of antibody--complement treated organisms. When viewed under the electron microscope the affected organisms were seen to change from filamentous structures to large circular structures with many tears or holes in the limiting membrane. Additional evidence that the membrane had been compromised was provided by the observation that complement-mediating killing was associated with release of 260 nm absorbing material, presumably nucleic acid.

C. Temperature Sensitive Mutants of M. pneumoniae - The benign nature of infection produced by ts mutants of M. pneumoniae in the hamster was confirmed. Initially we did not detect revertants of the ts mutants among isolates recovered from infected hamsters. More intensive search for such revertants revealed their occurrence in hamsters infected with several of the ts mutants which appeared to be most promising for use in a live M. pneumoniae vaccine. It was of interest that the hamsters which yielded revertants had a benign infection characterized by minimal pulmonary pathology. Although the ts mutants produced a limited attenuated infection it was sufficient to induce significant resistance to the pathologic effects of virulent M. pneumoniae organisms. Current efforts are aimed at identifying the ts mutant (or mutants) with a reversion rate that is so low that emergence of wild type organisms during infection will not occur with any appreciable frequency.

Significance of the Program to the Institute:

More sensitive serologic procedures for detection of M. pneumoniae antibody should prove helpful in illuminating several of the unexplained aspects of the biology of this organism. For example, now it should be possible to obtain a definite answer to the question of local antibody response. This is particularly important since previous studies using less sensitive serologic methods failed to identify local antibody, although challenge experiments in hamsters have demonstrated that local respiratory tract immunity plays a major role in resistance to M. pneumoniae. The possibility that prior silent infection with the organism sensitizes the host so that infection at a later date results in illness can now be investigated by the highly sensitive mycoplasmacidal and radioimmunoprecipitation antibody techniques. This represents a matter of some concern and has relevance to the direction to be taken in immunoprophylaxis of M. pneumoniae disease.

The development and characterization of ts mutants of M. pneumoniae appears to be one of the most promising approaches to prevention of disease caused by this organism. Current findings in this area offer some hope that the ts mutants will prove useful in the control of M. pneumoniae illness.

Proposed Course of the Project:

The newly developed highly sensitive serologic tests for *M. pneumoniae* antibody will be used in an effort to identify a local respiratory tract antibody response to the organism. These serologic methods will also be employed in seroepidemiologic studies designed to assess the significance of primary sensitization in pathogenesis of disease.

Genetic and pathogenesis studies with the ts mutants of *M. pneumoniae* will continue. Major emphasis will be given to the selection of the most suitable candidate mutants for evaluation in man.

Honors and Awards:

Invited speaker Workshop on "Mycoplasmatales as Agents of Disease, March 29, 1971, at NIH. Topic: "More Sensitive Methods for Detection of Antibody to *M. pneumoniae*".

Publications:

Brunner, H., Razin, S., Kalica, A.R., and Chanock, R.M.: Lysis and death of Mycoplasma pneumoniae by antibody and complement. *J. Immunol. 106*: 907-916, 1971.

Brunner, H., Horswood, R.L., Chanock, R.M.: More sensitive methods for detection of antibody to *M. pneumoniae*. Proceedings of the Workshop on the Mycoplasmatales as Agents of Disease. *J. Infect. Dis*., In press.

Chanock, R.M., Steinberg, P., Purcell, R.H.,: Mycoplasmas in human respiratory tract disease. In Sharp, J.T. (Ed.): *The Role of Mycoplasma and L-Forms of Bacteria in Disease*. Springfield, Ill., C.C. Thomas, 1970, Ch. 6 pp 110-136.

Cole, R.M., Popkin, T.J., Prescott, B., Chanock, R.M., and Razin, S.: Electron microscopy of solbulized *Acholeplasma laidlawii* membrane proteins reaggregated with *Mycoplasma pneumoniae* glycolipids. *Biochim. Biophys. Acta. 233*: 76-83, 1971.

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Purcell, R.H., Valdesuso, J.R., Cline, W.L., James, W.D., and Chanock, R.M.: Cultivation of mycoplasmas on glass. *Appl. Microbiol. 21*: 288-294, 1971.

Razin, S., Prescott, B., James, W.D., Caldes, G., Valdesuso, J.R., and Chanock, R.M.: Production and properties of antisera to membrane glycolipids of *Mycoplasma pneumoniae*. *Infection and Immunity 3*: 420-428, 1971.

Razin, S., Chanock, R.M., Rapport, M.M., and Graf, L.: Immunogenicity of cytolipin H aggregated with *Acholeplasma laidlawii* membrane proteins *Proc. Soc. Exp. Biol. Med*., In press, 1971.

Razin, S., Valdesuso, J., Purcell, R.H., and Chanock, R.M.: Electrophoretic analysis of cell proteins of T-strain mycoplasmas isolated from man. *J. Bact. 103*: 702-706, 1970.

Razin, S., Prescott, B., and Chanock, R.M.: Immunogenicity of *Mycoplasma pneumoniae* glycolipids. A novel approach to the production of antisera to membrane lipids. *Proc. Nat. Acad. Sci. U.S.A. 67*: 590-597, 1970.

Steinberg, P., Horswood, R.L., Brunner, H., and Chanock, R.M.: Temperature-sensitive mutants of *Mycoplasma pneumoniae.* II. Response of hamsters. *J. Infect. Dis*., In press, 1971.

Serial No. NIAID 70D
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

Previous Serial Number: Same

Principal Investigator: Dr. Robert H. Purcell

Other Investigators: Drs. Robert M. Chanock and Jerrold Lander

Cooperating Units:
Dr. Andrew Morrow (Surgery Branch, NHI, NIH)
Drs. Paul Holland, Harvey Alter and Paul Schmidt (Blood Bank, NIH)
Dr. John Walsh (Los Angeles, V.A. Hospital)
Dr. John L. Gerin (MAN Laboratory)
Dr. William London (NINDS)
Dr. Morris Suggs (CDC)
Dr. James Maynard (CDC)
Dr. Charles Cherubin (Harlem Hospital)
Dr. Lloyd Roseboom (Johns Hopkins)
Dr. Saul Krugman (N.Y.U.)
Dr. C.J. Peters (MARU)
Dr. Joseph Boggs (Children's Hospital, Chicago)
Dr. J. Anthony Morris (DBS)
Dr. Ralph Ziegler (Holloman Air Force Base)
Dr. James Sgouris (Michigan Department of Health)

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
P.O. D-130510-9	Dr. Purcell		Dr. J. Stein	$ 2,520
NIH 70-2034	Dr. Purcell		Mel Labs. Inc.	33,400
Interagency Agreement	Dr. Byrne	Oak Ridge Nat. Labs.	Drs. J. Liverman, N. Anderson	243,000
Interagency Agreement	Dr. Byrne	CDC	Dr. M. Suggs	19,800
PH 43-62-473	Dr. Chanock	Flow Labs.	Dr. Gilden	135,840

Serial No. NIAID 70D

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	60/12			60/12
Professional:	24/12	3/12		27/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies; (2) to develop and test *in vitro* procedures and animal systems for use in laboratory studies of viral hepatitis; (3) to study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigen.

Methods Employed:

(1) Clinical materials were collected from patients undergoing major heart surgery at NIH and from various animal and human populations through collaborative studies as described below.

(2) Hepatitis-associated antigen was purified by density banding in a cesium chloride gradient and by rate centrifugation in sucrose. Characterization was achieved with appropriate techniques, including analytical centrifugation, acrylamide gel electrophoresis and electrofocussing.

(3) Serum containing HAA was inoculated into various primate species, and the animals monitored for evidence of hepatitis for six months after inoculation.

(4) Serologic techniques, including complement fixation, agar gel diffusion, counterelectrophoresis, immunoelectrophoresis and radioimmunoassay were used for serologic studies.

Major Findings:

(1) Hepatitis Associated Antigen - Recently an abnormal protein (hepatitis associated antigen (HAA), Australia antigen) found in the serum of a high proportion of persons with leukemia, Down's syndrome and hepatitis, was found to be specifically related to infection with the virus of serum hepatitis. Furthermore, the partially-purified antigen was associated with virus-like particles 20-25 nm in diameter. HAA is believed by some to be the virus of hepatitis. Much of the work of this project has been directed toward the study of HAA.

(2) Serology - (A) Counterelectrophoresis: Early studies of HAA were performed using the agar gel diffusion technique for detecting the antigen. This technique was found to be relatively insensitive.

Subsequently we developed a complement fixation technique for detecting such antigen which was more sensitive than AGD but not suitable for large scale screening of blood or HAA such as might be done by blood banks. Modified immunoelectrophoretic techniques which were more sensitive than AGD but simpler to perform than CF were reported by several authors. Dr. Harvey Alter (Blood Bank Division, Clinical Center), in a cooperative study with our unit, developed an improved modified immunoelectrophoretic technique (counter electrophoresis, CEP) which was found to be more sensitive than other such techniques when it was employed to test a large panel of coded sera supplied by the National Research Council and Center for Disease Control. The modified technique is currently being adapted by other laboratories.

(B) Radioimmunoassay: Walsh, Yalow and Berson reported the development of a radioimmunoassay for HAA and anti-HAA which was more sensitive than all other serologic techniques. However, the RIA technique was cumbersome to perform and not useful for large serologic and epidemiologic studies requiring the testing of large numbers of serum specimens. We have modified the radioimmunoassay technique to make it suitable for large-scale seroepidemiologic studies. Furthermore, by using the appropriate reagents, antibody to HAA in specific immunoglobulin classes can be detected and measured. This new radioimmunoprecipitation (RIP) technique permits for the first time detection of anti-HAA following primary exposure to HAA-positive hepatitis.

(3) Epidemiology - (A) Longitudinal study of transfused patients: Early studies of hepatitis indicated that the most infectious period occurred prior to the appearance of jaundice. Serum specimens collected at that time would therefore be the most likely to yield viruses for cultivation. However, almost all field cases of hepatitis are diagnosed after jaundice appears and are therefore less desirable for collection of specimens. Patients who have undergone major heart surgery comprise a special high risk group for hepatitis. The risk of contracting hepatitis (probably a mixture of infectious and serum hepatitis and cytomegalovirus hepatitis) is directly proportional to the number of units of blood administered. Thus, many patients undergoing cardiac valve replacement at NIH (and who received as many as 20 to 30 units of blood) develop hepatitis during the post-operative period. This group of patients constitutes an ideal source of potentially infectious clinical material and epidemiologic data.

Although patients undergoing heart surgery at NIH come from all over the world, a significant proportion lives in the Washington and New York City metropolitan areas. It was felt that follow-up of patients from these two areas would yield the greatest amount of material with the least expense.

The method of following patients was as follows: all patients from the two surveillance areas were evaluated for suitability for inclusion in the study. The following criteria for inclusion were employed:

a) patients were at least 21 years of age, (b) major complications did not occur following surgery, (c) patients were accessible within the surveillance area and were fully cooperative and, finally (d) concurrence of the NIH heart surgery group. Patients were bled prior to surgery and at weekly intervals for three months and monthly intervals for 6 months. A blood specimen was also obtained after one year. Twenty to forty ml of blood were obtained at each bleeding, and serum was stored at -70°C. Serum specimens collected in New York were shipped to NIH at monthly intervals. Levels of SGO and SGP transaminase were determined on aliquots of all sera. Abnormal transaminase levels persisting for more than one week, and not related to cardiac failure or other obvious causes, were considered indicative of hepatitis. The patient's local physician was notified, and the patient was questioned about his illness when elevated transaminase levels were found. In addition to the patients described above, patients from other parts of the country were tested by their physicians for transaminase elevations at bi-monthly intervals and the results reported to NIH.

Since 1964 when the longitudinal study began, a total of 305 patients have been studied. The study has been divided into 3 phases: phase 1: 1964-1968, 110 patients. In this group, 42 persons developed hepatitis as determined by rise in serum transaminase values (attack rate = 38%). Of the 42 patients with hepatitis, 13 (31%) were jaundiced. When the relationship between the source of blood used at time of surgery and the development of hepatitis was studied, it was found that 42 of 82 (51%) persons who received predominantly (85%) blood from volunteers developed hepatitis. Such a difference in attack rates between the two groups was highly statistically significant. Thus, we have confirmed that the use of commercially obtained blood carries a much higher risk of hepatitis than does the use of blood from volunteer sources. Serial serum specimens from 32 heart surgery patients who were multiply transfused and who developed hepatitis and 32 similar patients who did not develop hepatitis were tested for HAA and anti-HAA. Four (12.5%) of the 32 patients who developed transfusion-associated hepatitis developed HAA in their serum as measured by CF and AGD; 32 patients who did not develop hepatitis were negative for HAA. In contrast two (6.3%) of the patients with hepatitis and 4 (12.5%) of those without hepatitis developed anti-HAA. Anti-HAA was not detected in sera from persons convalescing from HAA-positive hepatitis although serial sera were tested as late as six months to three years after onset of illness.

Phase 2: 92 patients were studied during this phase of the study; seven of them overlapped from Phase 1 and were included in both studies. Samples of blood from each donor unit transfused to the patients was saved and retrospectively examined for HAA and anti-HAA. 33% of the patients developed hepatitis; 43% of this hepatitis was icteric. 11 of 16 (69%) patients who received HAA positive blood developed hepatitis whereas only 19 of 76 (25%) patients who received HAA negative blood developed hepatitis. There was a significant difference in the frequency of hepatitis in the two groups (P=<0.01). The results suggest that an etiologic association exists between HAA in donor blood and the development of hepatitis in recipients of that blood, a finding in good agreement with the results of others. 52 of the 92 patients studied received commercially-derived blood and 40 patients received blood derived from volunteer sources. The frequency of hepatitis among patients who received commercial or volunteer blood was 50% and 10% respectively (P=<0.01). Thus, we confirmed our previous finding of an association between the use of commercially-derived blood and the development of hepatitis in the recipients of that blood. Furthermore, the association was not entirely the result of the greater frequency of HAA in commercially-derived blood when compared with volunteer blood (although such a greater frequency was found): the frequency of hepatitis among recipients of HAA-negative commercially-derived blood was four times greater than the frequency of hepatitis among recipients of HAA-positive and HAA negative volunteer blood. Thus, both commercially-derived blood and HAA-positive blood from whatever source were important factors in the development of hepatitis in blood recipients. Phase 3: 110 patients are being studied in phase 3, which is still in progress. During this phase of the project, only blood from volunteer sources has been transfused to patients. Furthermore, all such units were tested for the presence of HAA, and HAA-positive units were discarded prior to transfusion. Preliminary evidence suggests that the rejection of HAA-positive blood and the exclusive use of volunteer-derived blood has resulted in an 80% decrease in the frequency of hepatitis among the multiply-transfused patients studied.

(B) Hepatitis in the Far East: Hepatitis occurring among UN troops in Korea and the Far East during the Korean conflict was an important medical problem. This hepatitis was extensively studied and was thought to be classical infectious hepatitis. Recently, in a collaborative study with J. Anthony Morris (DBS), we examined serial serum specimens obtained from hepatitis patients in 3 geographic areas of the Far East (Japan, Korea, Okinawa) during the Korean conflict. Sixty percent of the patients were found to have HAA which was temporarily related to their hepatitis. Such a finding suggests that HAA-positive hepatitis, which is synonomous with serum hepatitis, may epidemiologically resemble infectious hepatitis in certain situations.

(C) Antibody to HAA in a human population: Sera from persons residing in the Washington Metropolitan area were tested for anti-HAA by the sensitive radioimmunoprecipitation (RIP) technique. Such antibody was found infrequently in infants and children but in increasing frequency in young and middle aged adults (almost 40% of middle aged adults had such antibody). The frequency diminished slightly in the elderly. Such a finding suggests that there is a high sub-clinical attack rate of HAA-positive hepatitis in the general population. It is unlikely that such a high attack rate can be explained by covert infection by parenteral means; it is therefore probable that HAA positive hepatitis is endemic in the population and is frequently transmitted by non-parental means, most likely by the oral route. Finding of antibody predominantly in adults is in good agreement with other studies in which HAA was found much more frequently in adults than in children. Sera from over 300 persons of all ages who were seen at Harlem Hospital, New York, are currently being studied in collaboration with Dr. Charles Cherubin of Harlem Hospital, New York. Preliminary evidence suggests an equally high frequency of anti-HAA among adults from Harlem.

(D) Anti-HAA in two Panamanian populations: In collaboration with Dr. C.J. Peters (MARU) sera from persons of all ages from two Panamanian Indian tribes (Chocoi and Guaymi) are being studied for age specific frequency of anti-HAA. The Chocoi and Guaymi tribes were selected for study because of the marked difference in HAA carrier rate between the two tribes. These studies are still in progress.

(E) Longitudinal study of hepatitis cases and family contacts: In a collaborative study with Dr. C.J. Peters (MARU) index cases of HAA positive and HAA negative hepatitis and their family contacts are being studied longitudinally. Such a study should help determine the relative communicability of these two forms of hepatitis and the inapparent attack rate of sub-clinical HAA-positive hepatitis.

(F) Relationship between pretransfusion antibody status and development of hepatitis in blood recipients: In a collaborative study with Dr. John Walsh (Los Angeles, V.A. Hospital) pre-transfusion and serial post-transfusion serum specimens were examined for anti-HAA with the RIA technique. Preliminary evidence suggests that patients with pre-existing RIA antibody did not develop HAA-positive hepatitis. However, some of the patients did develop HAA-negative hepatitis, probably caused by an agent distinct from the agent of HAA-positive hepatitis. In addition, a number of the patients developed a brisk antibody response to HAA following surgery. These patients had pre-existing RIA antibody to HAA and the pattern and the magnitude of their antibody response following transfusion resembled an anamnestic response.

(G) Anti-HAA in sera from primates: Sera from 81 chimpanzees from the chimpanzee colony, Holloman Air Force Base, New Mexico were obtained through the cooperation of Dr. Ralph Ziegler (Holloman AFB). 57% of the animals were found to have anti-HAA by RIP. Furthermore, the frequency of antibody increased with age; none of the animals under 2 years of age had such antibody whereas over 80% of animals 10 years old or older were positive for anti-HAA. Anti-HAA was found in both jungle-caught animals and animals born in captivity. Such a finding suggests that chimpanzees are highly susceptible to infection with HAA positive hepatitis.

Sera from 265 jungle-caught Rhesus monkeys were tested for anti-HAA by RIP. Two of these sera were definitely positive for anti-HAA by RIP. Thus, the Rhesus monkey may prove to be a suitable laboratory animal for the study of hepatitis.

(H) Demonstration of anti-HAA following long incubation period (MS-2) but not short incubation period (MS-1) hepatitis: In a collaborative study with Dr. Saul Krugman (NYU) serial serum specimens from children who were infected with MS-1 or MS-2 type hepatitis were tested under code for anti-HAA by RIP. All three children who developed MS-2 hepatitis with transient HAA antigenemia developed anti-HAA; one of three children who received the MS-2 inoculum and who developed chronic antigenemia developed such antibody. In contrast, none of 8 children who developed MS-1 hepatitis developed anti-HAA. These findings add further support to the belief that MS-1 and MS-2 hepatitis are caused by serologically distinct agents.

(4) Distribution of HAA in Fractions of Blood Fractionated by the Cohn Procedure - In a collaborative study with Dr. James Sgouris, Michigan Department of Health, and Dr. Paul Holland, Clinical Center Blood Bank, units of blood containing HAA, anti-HAA, or anti-complementary activity were fractionated by the Cohn procedure and the resultant fractions tested for HAA and anti-HAA by AGD, CEP, CF, and RIP. Preliminary evidence suggests that HAA was distributed in all fractions including fraction II (gamma globulin) and fraction V (albumin). Despite such a finding, extensive clinical experience indicates that properly prepared albumin and gammaglobulin are not icterogenic whereas the other fractions prepared from blood are known to be potentially icterogenic.

(5) Biophysical and Biochemical Studies of HAA - In a collaborative study with Dr. John Gerin (MAN), highly purified HAA was prepared by a series of isopycnic and rate zonal centrifugation procedures. The highly purified antigen was subjected to peptide analysis and found to consist of three discreet polypeptide moities.

Large scale centrifugal purification techniques were adapted to the purification of Australia antigen. The resultant large batches of purified antigen were found useful for the preparation of large quantities of specific anti-serum to HAA and for large scale biochemical analysis of the antigen.

(6) Detection of HAA in Individual Mosquitoes Artificially Fed on HAA Positive Blood - In a collaborative study with Dr. Lloyd Roseboom (Johns Hopkins) mosquitoes (albopictus species) were fed by the hanging drop technique on a blood meal prepared from HAA positive plasma. Mosquitoes were harvested at intervals of up to three weeks after feeding and tested for presence of HAA by the sensitive RIP technique. HAA was detected in mosquitoes up to five days after feeding. This technique may prove useful in attempts to detect HAA in mosquitoes and other arthropods from areas where HAA is highly endemic. Such arthropods may serve as a vector for HAA in highly endemic areas, either through the mechanical transfer of blood following interrupted feedings or following a developmental phase of the agent within the vector. However, such an association with arthropods has not been looked for under natural conditions.

(7) Antigenic Subtypes of HAA - Evidence from several laboratories suggest that multiple antigenic specificities exist on HAA particles and that these specificities may have epidemiological significance. To date, four antigenic specificities have been detected. We have confirmed the existence of such antigenic specificities and are currently preparing monovalent anti-sera to them.

(8) Use of Detroit-6 Tissue Culture Cells for the Isolation of Hepatitis Viruses - In a collaborative study with Dr. Joseph Boggs (Children's Hospital, Chicago) we are attempting to recover transmissible agents from acute phase serum specimens of persons with hepatitis, using the special clone of Detroit-6 cells supplied by Dr. Boggs. In addition, an isolate obtained from Dr. Boggs (Kirk-30 agent) is being studied in the Detroit-6 cells.

(9) Preparation of Reference Reagent Quality Hyperimmune Serum to HAA - In a collaborative study with the Reference Resources Branch, NIAID, Dr. Morris Suggs, Biological Reagents Branch, CDC, and Dr. John Gerin (MAN), large quantities of reference reagent quality anti-HAA were prepared in guinea pigs. The antiserum is being extensively tested by a panel of laboratories in cooperation with the WHO, Geneva.

(10) Attempts to Culture HAA in Vitro - We have attempted to culture HAA in human differentiated fetal liver cells cultured on polyethylene by the method of Fulton. In addition, HAA has been inoculated into organ cultures of human embryonic intestine. Harvested fluids from such cultures have been tested by CF for HAA and have been back-passaged into standard laboratory tissue culture cell lines (WI38, HEK, African green monkey kidney, Rhesus monkey kidney and HEP-2) and examined for CPE, hemadsorption and presence of interfering agents. In addition, we have attempted to intrinsically label any occult agents which might be present in such cultures with radioisotopes. To date these studies have failed to yield a transmissible agent.

(11) Attempts to Transmit HAA to Primates - Serum from patients with HAA-positive hepatitis was inoculated intravenously into 30 primates, including Rhesus, African green, and Cebus monkeys, and marmosets. A transaminase elevation was recorded in one African green monkey and one marmoset. In addition, several Rhesus monkeys developed RIP antibody to HAA. Serum from these animals is being passed to additional primates of the same species in an attempt to demonstrate serial transmission of a hepatitis agent. These primate studies are being carried out in collaboration with Dr. William London. In a collaborative study with Dr. James Maynard (CDC, Phoenix, Arizona) serum containing MS-2 hepatitis virus, obtained from Dr. Saul Krugman (NYU) was administered to chimpanzees found to be free of anti-HAA by RIP. These chimpanzees are currently being studied longitudinally.

Significance of the Program to the Institute:

Hepatitis represents one of the major unsolved infectious disease problems. The present program is consistent with the Institute's mission in that: (1) the sera from heart surgery patients constitutes one of the few recent collections of material which include pre-exposure and serial pre-icteric specimens from adults. Most of the sera collected during earlier volunteer studies have been exhausted, and additional volunteer studies with hepatitis material to generate virus-containing specimens are difficult to perform; (2) the recovery and characterization of hepatitis viruses and the subsequent development of vaccines is ultimately dependent upon the availability of specimens which have a high probability of containing these viruses; (3) the study of HAA, an antigen etiologically associated with serum hepatitis, will further our knowledge of this disease and provide important information necessary for the subsequent development of a vaccine.

Proposed Course of Study:

We plan to concentrate our studies of HAA in the following areas: (a) development of an animal model for HAA-positive hepatitis, (b) improvement in serologic tests for HAA and their use in seroepidemiologic studies, (c) extension of biophysical studies of HAA and anti-HAA interactions, (d) attempts to grow HAA in tissue and organ cultures, and (e) attempts to confirm and extend the studies of Krugman et al. which suggest that inactivated HAA is immunogenic and that antibody formed against such inactivated antigen is protective against serum hepatitis. In addition, we plan to extend techniques developed with HAA, where a serologic marker exists, to HAA-negative hepatitis specimens, where no marker has been detected.

Honors and Awards:

Participant, one day working seminar on Australia antigen, Center for Disease Control, Atlanta, Georgia, September, 1970.

Invited speaker at the symposium on "New Horizons in Infectious Diseases" sponsored by the Norfolk County Medical Society March, 1971. Topics: "Respiratory Viruses - Potential for Control" and "Hepatitis and the Australia Antigen".

Participated in the Simian Virus Workshop at the Southwest Foundation for Research and Education, San Antonio, Texas, April 26-28, 1971. Topic: "Unique Uses of Primates in Viral Research (slow viruses, Australia-antigen, Epstein-Barr Virus, others)."

Invited speaker International Seminar on Viral Hepatitis, International Children's Centre, Paris, June, 1971. Topic: "Seroepidemiologic studies of Australia antigen positive and negative hepatitis."

Invited participant in Round Table on Mycoplasmology, 71st annual meeting of the American Society for Microbiology, Minneapolis, Minn., May, 1971.

Publications:

Blumberg, B.S., Byrne, R.J., Chanock, R.M., Cockburn, W.C., Kono, Y., Koza, J., McCollum, R.W., Menache, D., Pennttinen, K., Purcell, R.H., Taylor, P.E., Wewalka, F., and Zuckerman, A.J.: Viral hepatitis and tests for the Australia (hepatitis-associated) antigen and antibody. Bull. World Hlth. Org. 42: 957-992, 1970.

Gerin, J.L., Holland, P.V., and Purcell, R.H.: Australia antigen: Large-scale purification from human serum and biochemical studies of its proteins. J. Virol. In press, 1971.

Hill, D.A., Walsh, J.H., and Purcell, R.H.: Failure to demonstrate circulating interferon during incubation period and acute stage of transfusion-associated hepatitis. Proc. Soc. Exp. Biol. Med. 136: 853-856, 1971.

Lander, J.J., Alter, H.J., and Purcell, R.H.: Frequency of antibody to HAA as measured by a new radioimmunoassay technique. J. Immunol. 106: May, 1971.

Lander, J.J., Giles, J.P., Purcell, R.H., and Krugman, S.: Viral hepatitis, type B (MS-2 strain): detection of antibody following primary infection. New Eng. J. Med., In press, 1971.

Purcell, R.H., Walsh, J.H., Holland, P.V., and Morrow, A.G.: Studies of transfusion-associated hepatitis. In Sanders, M., and Schaeffer, M., (Eds.): Viruses Affecting Man and Animals, St. Louis, Missouri, Warren H. Green, Inc., 1971, pp. 274-283.

Serial No. NIAID 70E
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biological and Biophysical Studies of Rhinoviruses

Previous Serial Number: Same

Principal Investigators: Dr. John C. Perkins
Dr. Robert F. Buscho

Other Investigator: Dr. Albert Z. Kapikian

Cooperating Units: None

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309	Dr. Chanock	Flow Labs	Dr. Gilden	$ 135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	42/12			42/12
Professional:	18/12			18/12
Other:	24/12			24/12

Project Description:

Objectives: (1) To develop and characterize temperature sensitive (ts) conditional lethal mutants of rhinoviruses for potential use in a live attenuated vaccine; (2) to study the local respiratory tract antibody response to rhinovirus antigens and to develop methods for potentiation and prolongation of this type of response.

Methods Employed:

(1) For the genetic studies a strain of rhinovirus 1A was plaque purified by three successive plaque passages at 37°C. The virus was then grown in the presence of $10^{-2.0}$M 5-fluorouridine (5FU) or 5 to 7 µg proflavine per ml. Because of the high rate of reversion exhibited by the 5FU mutants treatment with both mutagens was performed in an attempt to obtain stable mutants. A ts mutant was defined as a virus clone that grew to a titer of at least 10^3 higher at the permissive temperature (32°C) than at the non-permissive temperature (38°C).

(2) Rabbits were given 5 inactivated rhinovirus antigens simultaneously. In addition, one group of rabbits also was given a synthetic polyribonucleotide (poly I-poly C) together with the viral antigens. Serum and nasal washings were collected periodically to determine if the polyribonucleotides enhanced the local or serum antibody response.

(3) After extensive safety tests in guinea pigs had shown that poly I-poly C was not toxic when given together with rhinovirus antigen, 9 volunteers were given an inactivated rhinovirus type 13 (RV13) vaccine together with the synthetic polynucleotide intranasally. A control group of 9 men received the vaccine with saline. Serum and nasal washings were collected periodically to determine if the polyribonucleotide enhanced the local or serum antibody response.

Major Findings:

(1) Evaluation of a 5FU Induced ts Mutant in Volunteers - The most stable of the 5FU induced ts mutants was given to 14 seronegative volunteers by the intranasal route. The men received either $10^{4.7}$ or $10^{5.0}$ $TCID_{50}$. Thirteen of the 14 men became infected and 9 men developed typical rhinovirus common cold illness. This response indicated that the mutant did not possess the desired degree of attenuation. Virus isolates were analyzed for evidence of wild type revertants and only 2 men were found to shed this type of virus. Since neither of these men became ill it seems clear that reversion of the mutant to the wild type was not responsible for the virulence of the ts mutant suspension given to the volunteers.

(2) Characterization of the 5FU ts Mutants - Temperature shift experiments indicated that the ts defect(s) in 2 of the mutants affected late events in the virus replicative cycle.

The 15 mutants isolated last year (5 from untreated virus and 10 from 5FU treated virus) were studied under carefully controlled conditions of constant temperature. Each of the mutants was markedly restricted in plaque forming ability at 38°C. At 37°C there was little restriction evident for any of the mutants.

(3) Potentiation of Local Antibody Response by Poly I-Poly C - Last year we found that poly I-poly C potentiated the serum antibody response of rabbits to inactivated viral antigen given by the intranasal route. In an attempt to extend these findings 5 rhinoviruses (types 1A, 8, 13 19 and 22) which had been grown to a titer of $10^{6.3}$-$10^{7.5}$ pfu per ml in HeLa cell spinner culture were given simultaneously by the IN route to rabbits. A group of rabbits also received poly I-poly C IN at the same time. Nasal washes and sera were collected and are now being analyzed for antibody titer by a recently developed microplate plaque reduction technique.

(4) Attempt to Potentiate Local Antibody Response in Man - In an effort to potentiate and prolong the nasal antibody response to rhinovirus antigens, 18 male volunteers, who lacked detectable serum and nasal secretion neutralizing antibody to rhinovirus 13 (RV13), were given inactivated type 13 virus vaccine IN. One-half the group received inactivated RV13 vaccine plus poly I-poly C (two immunizations one week apart) and the other 9 men received the RV13 vaccine plus saline IN. Biochemical and hematological studies on both groups revealed no apparent toxicity from the poly I-poly C, and none of the men showed any signs or symptoms of an allergic reaction to either the vaccine or the poly I-poly C. An extensive testing program had been carried out in guinea pigs prior to approval for use of the vaccine and poly I-poly C together in humans. We were unable to demonstrate any sensitization of the guinea pigs by either the vaccine or the poly I-poly C.

Six weeks after the last dose of vaccine, the poly I-poly C group had local and serum neutralizing antibody titers which were somewhat higher than the men who received vaccine without poly I-poly C. Although it is too early to draw definite conclusions, it appears that poly I-poly C did have some effect on the nasal antibody response to a rhinovirus antigen but the response was not very great.

Significance of the Program to the Institute:

The use of conditional lethal mutants represents a promising approach to the development of a live attenuated vaccine. In addition, studies on naturally occurring and chemically induced ts mutants may help to explain the extreme antigenic lability of the rhinoviruses which now comprise at least 90 distinct serotypes.

One of the major needs of the Institute's vaccine program involves potentiation of the local respiratory tract antibody response since this type of antibody appears to be the major determinant of resistance to rhinovirus and myxovirus infection. The finding that a synthetic polyribonucleotide enhances the local antibody response may prove helpful in the development of procedures for vaccination by the nasal route.

Proposed Course of Study:

Mutants derived from virus subjected to 2 separate chemical mutagens will be safety tested and evaluated in volunteers. Mutants with a shut-off temperature of 37°C will be sought with the hope such viruses will cause little or no illness and yet induce resistance to naturally occurring disease. The studies in rabbits and human volunteers on the ability of poly I-poly C to enhance the local immune response will continue. New inactivated rhinovirus vaccines will soon be available so that multiple vaccines can be tested in man.

Honors and Awards: None

Publications: None

Serial No. NIAID 72A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: New Techniques for Recovery of Respiratory Tract Viruses

Previous Serial Number: Same

Principal Investigators: Dr. Robert A. Bucknall
Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: None

Contract Support:

Contract Project Number	Project Officer (NIAID)	Contractor	Contractor's Project Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs.	Dr. Gilden	$135,840

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	19/12		5/12	24 /12
Professional:	8/12		5/12	13 /12
Other:	11/12			11 /12

Project Description

Objectives: (1) To recover new viruses from patients with respiratory disease; (2) to characterize such agents and study their biologic and biophysical properties; (3) to determine the importance of such viruses as etiologic agents in human respiratory disease.

Methods Employed:

Material studied by new techniques has been derived from investigations of respiratory disease conducted by this laboratory and from other studies performed in cooperation with other institutions. Such material includes specimens (nasopharyngeal washings and/or throat swabs and acute and convalescent sera) from patients at Children's Hospital in D.C., Johns Hopkins Hospital, D.C. General Hospital, Junior Village, and from volunteers in the NIH Common Cold Study. Specimens obtained from patients in the NIH Common Cold Study have been studied most intensively; the specimens are first examined by routine tissue culture methods, and only those which fail to yield viruses are studied further in experimental culture systems -- such systems include tissue cultures and human embryonic tracheal (HET) organ cultures. The use of new culture systems has been especially fruitful in the recovery of virus strains belonging to the human coronavirus group of viruses.

Fifteen of the 23 known coronavirus strains were first recovered in this laboratory employing new culture systems. However, extensive study of the coronaviruses has been hampered by the lack of convenient and suitable methods for their propagation. Nine of the 23 coronavirus strains were originally isolated in HET organ cultures only, while the remaining 14, all of which were similar to a prototype strain designated 229E, were isolated with difficulty in monolayer tissue cultures. Since the coronaviruses appear to be important etiologic agents of adult upper respiratory tract illnesses continued intensive efforts have been made to propagate these agents in a suitable tissue culture system to facilitate epidemiologic, antigenic, biological, and biophysical studies of these agents. In addition, various studies of coronavirus strains 229E, which was originally recovered in tissue culture,and OC43, which was originally isolated in HET organ culture but later adapted to suckling mice and monkey kidney tissue cultures, were performed since suitable tissue culture systems were available. Some of these studies are described below.

Major Findings:

Hemadsorption by Coronavirus Strains OC43 and Adaptation of OC43 Virus to Additional Cell Cultures - Erythrocytes from 5 animal species and man were studied for hemadsorption in BS-C-1 cultures infected with OC43 virus which had been previously adapted to suckling mice and then to BS-C-1 cell cultures. Rat and mouse erythrocytes hemadsorbed readily, adult chicken erythrocytes minimally, vervet MK and human "O" cells minimally and inconsistently and guinea pig cells not at all. In early stages of infection, clusters of red blood cells arranged in ovals, rosettes and triangular shapes adhered to 10-25% of the cell sheet. Later, the entire cell monolayer was covered with firmly adsorbed erythrocytes. In further experiments, hemadsorption was also demonstrated in OC43 virus infected human embryonic kidney (HEK), rhesus monkey kidney (MK), and human diploid cell strain (HDCS) WI38 cell cultures. This marked the first adaptation of suckling mouse and BS-C-1 passaged OC43 virus in HEK and HDCS WI38 cell cultures. In all cell cultures studied, hemadsorption appeared several days to one week before cytopathogenic effect (CPE). Prevention of hemadsorption by specific OC43 hyperimmune antisera was demonstrated in conventional neutralization tests using the complete absence of hemadsorption as the measure of neutralization. Hemadsorption was also used as the indicator system for OC43 virus neutralization tests with human sera. In such tests, 2 individuals with significant 229E CF antibody rises (one of whom yielded 229E virus) did not develop significant OC43 virus neutralizing antibody rises while 4 of 7 with such CF antibody rises to OC43 virus did; one of the 4 individuals who developed a neutralizing antibody rise in the latter group also yielded OC43 virus. Hemagglutination-inhibition (HI) tests for OC43 virus were also performed and it was found that only 3 of 7 patients with a CF antibody rise to OC43 virus developed four-fold or greater increases in serum antibody in the HI test, and these individuals also had a rise by the neutralization-hemadsorption technique. The serologic data suggest that OC43 antigen may react more broadly in the complement fixation test with antibody induced by other coronaviruses thereby accounting for the greater reactivity (and lesser specificity) of the complement fixation in comparison to the HI and neutralization techniques in these limited studies. It was also of interest that all 4 individuals with OC43 virus neutralizing antibody rises possessed neutralizing antibody in acute phase sera. Further studies should reveal whether or not the pattern of OC43 virus infection in adults is similar to that of respiratory syncytial and parainfluenza 3 viruses both of which characteristically produce reinfection in adults.

Intracellular Development and Mechanism of Hemadsorption of OC43 Virus-
The intracellular development of OC43 virus grown in HDCS WI38 cells was studied by electron microscopic techniques. These studies revealed that OC43 virus, like coronaviruses IBV, 229E and MHV, is formed in intracellular vesicles and not by budding at the external cell membrane in the manner of the myxoviruses. No clear evidence of budding by OC43 viruses into the cytoplasmic vesicles was observed. The mechanism of the hemadsorption reaction was also studied and it was found that it resulted from the accumulation of progeny virus on the outside surface of the cells in sufficient quantity to bind the rat red blood cells.

Study of Physical and Biological Properties of OC43 and 229E Viruses -
Study of some of the physical and biological properties of OC43 virus was feasible since the hemadsorption reaction provided a simple indicator system for virus multiplication. Inactivation studies of OC43 and 229E viruses by heat, acid and ultraviolet (UV) light were performed. In the absence of calf serum, they had similar thermal (33°C and 37°C), acid and UV inactivation rates. However, when the viruses were grown in the presence of serum, the UV inactivation rates were reduced and evidence suggested that this resulted from viral aggregation. The UV inactivation rate of serum-free OC43 and 229E viruses was found to be similar to that of influenza A2 suggesting that the genomes were of comparable size.

Studies on the growth of OC43 and 229E viruses at various temperatures revealed that OC43 virus grew well from 32-34°C although virus growth was delayed at 32°C; at 35°C virus growth was reduced by 90% and at 36°C no virus growth could be detected. 229E virus grew best at 32°-33°C and was progressively inhibited at 34°C, 35°C and 36°C; at 37°C no virus growth was detected. The nature of the temperature sensitive (ts) event was investigated by using temperature shift techniques to establish at what point in the virus growth cycle the ts event occurred and how long it lasted. These studies revealed that the ts event began 10 hours after infection at 33°C and extended beyond the time when virus release started at 12 hours. The temperature sensitive event was probably the synthesis of the viral structural proteins or some step immediately prior to this.

Attempts to Adapt Human Coronaviruses to Grow in Conventional Tissue Culture Systems - Efforts to adapt some of these fastidious agents to numerous tissue cultures were continued. Coronavirus strains OC16, OC44 and B814 were grown in HET cultures and seeds prepared which showed typical coronavirus particles when examined by electron microscopy. These infectious seeds together with 229E and OC43 virus (which acted as markers) were inoculated into a total of 42 types of tissue culture cells. After 2 weeks or when the cultures degenerated, fluids were harvested without freezing and thawing and inoculated into fresh cultures of homologous cells. When these cultures showed signs of degenerating, the fluids were examined under the electron microscope for coronavirus particles, and the cell sheets were tested for hemadsorption with rat red blood cells. In addition, tubes of each cell type were infected with 100 $TCID_{50}$ of OC43 and 229E viruses and their growth detected by hemadsorption and CPE respectively. There was no evidence that strains OC16, OC44, and B814 had grown in any of the 42 types of tissue culture cell tested. OC43 and 229E viruses grew best in human embryonic fibroblast cultures and not at all in a wide range of continuous human lines and a variety of animal cells.

Additional attempts were also made to propagate these fastidious agents in the following ways: (1) HET rings infected with OC16, OC44, and 229E viruses and shown to be producing virus by electron microscopic examination of the culture fluids, were placed directly on cell sheets of HDCS WI38 cells. In the case of 229E, the WI38 cells showed CPE after 4-5 days as expected. The sheets with the other infected HET rings did not develop CPE for 28 days and fluids from them were passaged four times in WI38 cells; no evidence of virus growth was detected; (2) multiple passages of OC16, OC44, B814 and HET-grown OC43 (as a possible marker) were attempted in HDCS WI38, whole human embryo and primary HEK cell cultures all of which were sensitive to 229E and tissue culture adapted OC43 viruses. After 7 fourteen day passages in WI38 and whole human embryo cells, and 12 seven day passages in HEK cells, there was no evidence of CPE or hemadsorption and in addition no coronavirus particles were detected in the final culture fluids by electron microscopy; (3) stimulated by a recent report by Sturman and Takemoto of NIAID that the growth of 4 MHV strains was enhanced in polyoma-transformed mouse embryo cell cultures, attempts were made to adapt human coronaviruses to an SV40 transformed WI38 cell strain culture designated Va-2. OC16 and OC44 were passaged several times in tube cultures of Va-2 cells, but virus was not detected; 229E virus grew in these cultures but OC43 virus did not.

Factors Affecting the Growth of Human Coronavirus Strain 229E in Organ Culture - The results from this limited study of 14 human embryonic trachea suggest that the sensitivity of HET cultures to 229E virus is not affected by age, sex, or race of the fetus from which the trachea is obtained. It was noteworthy, however, that one of the 14 tracheas used in the study grew 229E virus very poorly and another not at all. Both tracheas were in good condition and no factor in the medical history of the mother was evident that could account for this reduced virus sensitivity; from these studies it would appear that when human respiratory viruses are isolated or passaged in HET cultures, a suitable marker virus should be included in each experiment to establish the sensitivity of the organ culture used.

Immune Electron Microscopy for Detection of Coronaviruses - Routinely in our laboratory, nasal washings which fail to yield viruses in conventional tissue culture are passed 3 times in HET organ culture. The fluids from the third passage are harvested, clarified, and concentrated by ultracentrifugation, negatively stained, and examined for coronavirus particles by electron microscopy. However, it is known that following the addition of specific antiserum to a virus preparation, aggregates of the virus particles will form with visible antibody bridges linking the particles. Previously difficult to visualize viruses such as rubella (Best et al) and the rhinoviruses (Project # 70B)have been visualized clearly by this technique. Therefore, a variation in our routine examination of third passage organ culture harvests was made in an attempt to take advantage of the aggregation of virus particles by specific sera. The third passage HET harvest was incubated with the patient's convalescent serum for one hour at room temperature and then the mixture was concentrated by ultracentrifugation, negatively stained, and examined under the electron microscope. These studies have been initiated recently and will be pursued intensively in the coming year.

Seroepidemiologic Studies of Coronaviruses - Such studies were reported in previous annual reports. Additional seroepidemiologic studies are reported in Projects #66A and 69C.

Significance of Program to NIH:

The search for new agents using the techniques outlined above is of importance in identifying possible etiologic agents in respiratory disease and as an initial step toward the ultimate goals of prevention and therapy. The discovery of new agents such as those described above attests to the value of this endeavor. Further study of the biologic and biophysical nature of these viruses, and the epidemiology and pathogenesis of diseases associated with them can be expected to bring us closer to these goals.

Proposed Course of Project:

The search will continue for new agents from patients with respiratory disease using the techniques outlined in this report, and any new systems which appear to be profitable. Further epidemiologic surveys will be conducted to assess the role of coronavirus infection in respiratory tract illness. In addition, further attempts will be made to adapt the organ culture strains to tissue culture and to devise new methods for their study.

Honors and Awards: None

Publications:

McIntosh, K., Kapikian, A.Z., Turner, H.C., Hartley, J.W., Parrott, R.H. and Chanock, R.M.: Seroepidemiologic studies of coronavirus infection in adults and children. Amer. J. Epidem. 91: 585-592, 1970.

McIntosh, K., Bruckova, M., Kapikian, A.Z., Chanock, R.M. and Turner, H.C.: Studies on new virus isolates recovered in tracheal organ culture. Annals of the New York Academy of Sciences 174: 983-989, 1970.

Bruckova, M., McIntosh, K., Kapikian, A.Z., and Chanock, R.M.: The adaptation of two human coronavirus strains (OC38 and OC43) to growth in cell monolayers. Proc. Soc. Exp. Biol. Med. 135: 431-435, 1970.

Serial No. NIAID 72B
1. Infectious Diseases
2. Respiratory Viruses and Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Respiratory Viruses and Mycoplasmas in Human Volunteers

Previous Serial Number: Same

Principal Investigators:
Peter F. Wright, M.D.
Elias G. Chalhub, M.D.
John C. Perkins, M.D.
Brian Murphy, M.D.
Robert F. Buscho, M.D.
Robert M. Chanock, M.D.

Cooperating Units:
Dr. Louis Potash, Flow Laboratories
Dr. Richard Hornick and Dr. Y. Togo, University of Maryland, School of Medicine
Dr. Robert H. Parrott, and Dr. H.W. Kim, Children's Hospital of D.C.

Contract Support:

Contract Number	Proj. Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 135,840
PH 43-66-63	Dr. Chanock	Univ. Md.	Dr. Hornick	63,600
Interagency Agreement	Dr. Chanock	Lorton Ref.		64,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	81/12			81/12
Professional:	45/12			45/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To determine if a temperature-sensitive (ts) mutant of parainfluenza type 1 (Para 1) is attenuated for man and to study the secretory antibody response to infection with ts mutant; (2) to determine if infection with one paramyxovirus (type 1 parainfluenza) induces short term heterologous resistance to another biophysically similar virus (RS); (3) to evaluate temperature sensitive mutants and recombinants of influenza A virus for attenuation in adult volunteers; (4) to characterize the nasal secretory antibody response induced by intranasal vaccination with an inactivated rhinovirus vaccine; (5) to investigate further the role of nasal secretory neutralizing antibody in protection against experimental rhinovirus infection. Since it has been shown in previous studies that intranasal vaccination induces resistance to experimental challenge we were particularly interested in whether this immunity persisted beyond the immediate post-vaccination period.

Methods Employed:

(1) Parainfluenza Type 1 - The type 1 parainfluenza virus mutant studied was derived by growth of virus at 26°C in African green monkey kidney (AGMK) tissue culture. The 20993 strain of type 1 virus was passaged 20 times in AGMK cultures which were incubated at 26°C. Subsequently, the virus was purified by 3 successive terminal dilutions. The resulting virus population exhibited partial restriction in growth at 38°C and 39°C when compared to the parent virus from which it was derived. The mutant which was selected by the terminal dilution procedure was apparently a minority component of the virus population because prior to the terminal dilution, the virus did not exhibit temperature sensitivity. Extensive safety tests at Flow Laboratories failed to reveal the presence of adventitious agents.

The subjects for these studies were volunteers from the District of Columbia Reformatory in Lorton, Virginia. They were selected on the basis of general good health and low level of nasal neutralizing antibody titer to Para 1 (1:2 or less). Informed consent was obtained prior to participation in the study. The men were maintained in isolation in a dormitory at Lorton Reformatory for 2 weeks. Volunteers were examined daily by two physicians, and cultures, respiratory secretions, and blood samples were taken when appropriate. The men were inoculated intranasally with $10^{4.8}$ $TCID_{50}$ with one-half of the inoculum administered as a coarse droplet aerosol (DeVilbiss atomizer), while the remainder was pipetted slowly into the nares. Nasopharyngeal washings for virus isolation were collected in veal infusion broth daily and inoculated into rhesus monkey kidney roller tube cultures. Cultures were examined for presence of a hemadsorbing virus every 5 days and discarded after 20 days if negative. Virus isolates were identified by the hemadsorption-inhibition test. Serum antibody and nasal antibody levels were determined by the microtiter-neutralization test developed in this laboratory.

(2) Influenza Virus - The influenza virus studies were performed in a similar manner at the Lorton Reformatory or the Maryland House of Correction, Jessup, Maryland. The influenza virus suspensions used were subjected to extensive safety tests and adventitious agents were

not detected. Volunteers who lacked detectable serum neutralizing antibody for the influenza A2/Hong Kong/1968 virus were selected for participation in these studies. Approximately 10^5 pfu of wild type or ts mutant were given by the method described above.

(3) Inactivated Rhinovirus Vaccine - A group of 10 volunteers were vaccinated intranasally with a formalin inactivated rhinovirus 13 vaccine. Nasal wash and serum specimens were obtained from the volunteers and analyzed for RV13 neutralizing activity. The protective efficacy of the vaccination was tested six months following vaccination when the intranasal vaccinees along with ten additional seronegative volunteers were challenged with live RV13. The clinical response of these two groups to challenge was compared.

An additional study with these volunteers was initiated to see whether immunologic memory is a characteristic of nasal secretory antibody production. A group of volunteers who had prior experience with RV13 antigens (from intranasal vaccination or live virus challenge or both) were given vaccine intranasally along with a group of newly recruited RV13 seronegative volunteers. The nasal wash and serum neutralizing antibody response to vaccination was then analyzed in these volunteers.

Major Findings:

A. Cold-Adapted Parainfluenza Type 1 Virus - The *in vivo* behavior of this mutant in hamsters indicated marked restriction of growth in the lungs and trachea, suggesting that it was suitable for evaluation in adult volunteers. Nineteen men were selected as candidates for this study because they possessed low or undetectable levels of nasal secretion antibody for type 1 virus (titer 1:2 or less). $10^{4.8}$ $TCID_{50}$ of virus was given to 16 of the 19 adult volunteers. Ten of the 16 men became ill with a mild to moderate upper respiratory tract illness. Thirteen of the 16 men shed virus from 3-9 days following administration of the type 1 preparation. Three of the 19 men were purposely not given virus and virus was not recovered from these volunteers, suggesting that transmission of the mutant does not occur with facility. A summary of virus recovery, immune response, and illness is shown in the following table.

Group	No. of men	No. who shed virus	Number of men with serum antibody response*		Upper respiratory illness
			CF	Neut.	
Men given virus	16	13	1	12	10
Men not given virus	3	0	0	0	0

* Four-fold or greater rise.

Twelve of the 16 men who received virus developed a four-fold or greater rise in serum neutralizing antibody titer while 1 of the men in this group also developed a rise in CF antibody. The analysis of nasal antibody response has not been completed.

The viral isolates were grown in tissue culture along with the original mutant at a permissive temperature (32°C) and a restrictive temperature (38°C or 39°C). The "mutant" virus given the volunteers did not grow at the restrictive temperatures, while each of the viral isolates grew at both the permissive and restrictive temperatures indicating a high rateof reversion *in vivo*. These findings indicated that the mutant was not a candidate for continued evaluation in subjects of progressively younger age.

B. Attempt to Demonstrate Heterologous Resistance Following Type 1 Parainfluenza Virus Infection - Successful infection of 13 of the 16 volunteers who received the cold adapted type 1 parainfluenza virus ts mutant provided an opportunity to re-examine the phenomenon of heterologous resistance induced by respiratory infection. Previously heterologous protection had been demonstrated by workers in LCI, NIAID, following rhinovirus or Coxsackie virus infection. This protection extended for at least 5 weeks in 2 studies; however, other investigators were unable to confirm these findings. We were interested in determining if such non-specific resistance could be demonstrated between two paramyxoviruses. We chose RS virus as the challenge virus since we had shown last year that the ts-1 mutant of RS virus induced significant resistance to wild type RS virus, although administration of the mutant had not stimulated detectable increases in either local respiratory tract or serum antibody. For this reason we wondered whether or not the protective effect of the ts-1 mutant of RS virus was antigenically specific. To answer this question men who had received the type 1 parainfluenza virus were challenged 45 days later with $10^{4.1}$ pfu of wild type RS virus and their response compared to men who received the same wild type challenge 45 days after the ts-1 mutant of RS virus. In addition a group of men who had not received either mutant were given the wild type virus. The results of the challenges are shown in the following table.

Response of Adult Volunteers to Nasopharyngeal Instillation of Wild Type RS Virus

RS virus inoculum	Challenge 45 days previously	No. of men	Number with indicated findings				Upper resp. illness associated with RS infection
			Virus excretion	Immunologic response			
				Serum neut. or CF antibody	Nasal wash neut. antibody	Total	
Wild type ($10^{5.0}$pfu)	none	8	6	3	5	5	5
Wild type ($10^{5.0}$pfu)	RS ts-1 mutant	7	1	0	0	0	0
Wild type ($10^{4.1}$pfu)	Para 1 ts mutant	8	6	3	5	5	5

These results show a complete lack of heterologous protection when the men previously infected with type 1 parainfluenza virus were challenged with RS virus. This lends further support to the thesis that administration of the RS ts mutant had induced specific resistance to RS virus.

C. Evaluation of Temperature-Sensitive (ts) Mutants of Influenza Virus in Volunteers - The two ts mutants of influenza A_2 (1965) were evaluated in volunteers with the lowest possible levels of serum antibody. Unfortunately none of our volunteers had less than a moderate titer of antibody. This situation made it difficult to assess the state of attenuation of the mutants. Ts-1, which had a "shut-off" temperature of 37°C and which was markedly restricted in the hamster, was administered intranasally (IN) in a dose of 10^5 pfu to 12 men with serum neutralizing antibody titers of 1:32 - 1:64. Virus was not recovered nor did illness occur. However, serologic studies indicated that 8 of the 12 men had been infected; there were four-fold rises in either nasal or serum neutralizing antibody or both. Identical results were obtained with ts-2, which "shut-off" at 39°C and which was only moderately restricted in the hamster; 8 of 12 men developed an immunologic response without shedding detectable virus or becoming ill. These men likewise had a high titer of neutralizing antibody (1:64 - 1:1024) prior to administration of 10^5 pfu of ts-2. A parallel passaged wild type virus was given to a group of 10 volunteers for comparative purposes. 10^5 pfu were given intranasally to men who had serum neutralizing antibody titers of 1:64 - 1:1024. None of the men shed virus or became ill; however, 5 men developed an antibody response. These findings clearly demonstrated that the ts mutants could infect and elicit an antibody response in volunteers who had had previous experience with the major antigens of A_2/65 influenza. However, the critical question of attenuation could not be answered because in our volunteers, who were selected on the basis of lowest possible levels of serum antibody, the wild type virus did not produce disease.

When it became clear that the ts mutants of the 1965 influenza A virus could not be assessed for level of attenuation, another approach was taken. Since 10-20% of our volunteer population lacked serum antibody for the Hong Kong influenza A virus we produced ts recombinants with the hemagglutinin of Hong Kong and the ts defect(s) of influenza A_2/1965 (see Project 70A). A Hong Kong-ts-1 recombinant with a shut-off temperature of 37°C was given IN to 13 volunteers (at Lorton Reformatory) who lacked serum neutralizing antibody for the Hong Kong virus (inoculum of 10^5 pfu). We were unable to detect evidence of infection and thus it appeared that this recombinant was over-attenuated.

Another Hong Kong recombinant which was derived from mixed infection with the ts-2 mutant of influenza A_2/1965 was given to 9 volunteers by the IN route (10^5 pfu). These men also lacked serum neutralizing antibody for the Hong Kong virus. Each of the men developed a serum neutralizing antibody response and/or shed virus. Response to infection was minimal; it was characterized by nasal stuffiness and other symptoms of upper respiratory disease without fever. Influenzal illness did not occur. These findings indicated that the Hong Kong ts-2 recombinant, with a shut-off temperature of 39°C, was attenuated, but the level of attenuation was not sufficient to consider using this recombinant in a live vaccine. Serum antibody response to the recombinant (mean serum antibody titer during convalescence of 1:13) was lower than that seen with wild type Hong Kong virus (1:130). The last isolate recovered from each of the 8 volunteers who shed virus was characterized for temperature sensitivity. In each instance the isolate retained the ts property of the input recombinant, indicating that the recombinant ts mutant was stable in vivo.

Next we investigated the ability of volunteers who received the Hong Kong ts-1 or ts-2 recombinant to resist challenge with wild type virus. As expected from our failure to detect evidence of infection with the ts-1 recombinant, men who received this virus did not exhibit resistance to wild type virus. In contrast, previous administration of the ts-2 recombinant altered the response of volunteers to subsequent challenge with the wild type virus. There was a significant reduction in shedding of wild type virus. In addition there was a reduction in occurrence of influenzal illness; however, this difference was not statistically significant because of the low frequency of such disease in the control group of volunteers who had not been given ts-1 or ts-2 prior to wild type virus challenge. The serum antibody response of the men who received wild type virus after prior administration of Hong Kong ts-1 or ts-2 recombinant is shown in the following table.

Prior administration* of 10^5 pfu of	Challenge virus**	Number of men in group	Geometric mean (reciprocal) serum neutralizing antibody on indicated day after challenge with wild type HK virus				
			0	7	14	21	28
A2/HK/ts-1	A2/HK wild type	8	4.4	5.4	42	59	71
A2/HK/ts-2	A2/HK wild type	5	12	31	36	85	85
---	A2/HK wild type	8	4	5	29	81	120

* 60 days before challenge with wild type Hong Kong virus.
** 10^5 pfu

The early response 7 days post-challenge exhibited by the prior recipients of the Hong Kong ts-2 suggests that these men had been primed immunologically to respond to the antigens of the Hong Kong virus. Men who had received the Hong Kong ts-1 recombinant did not exhibit such an accelerated response; this is consistent with the other evidence that this recombinant failed to infect.

D. Antibody Response Following Primary Intranasal Administration of Inactivated RV13 Vaccine - Each volunteer developed a 4-fold or greater rise in serum and nasal wash RV13 neutralizing antibody 30 days after being immunized. Serum and nasal wash neutralizing activity reached its highest level in the first specimen collected four weeks following the last dose of vaccine and subsequently declined. The nasal neutralizing activity seemed to decline more rapidly than the serum antibody titer.

E. Specificity of Nasal Secretory Antibody Induced by RV13 Vaccine - In order to evaluate the specificity of nasal secretory neutralizing antibody induced by rhinovirus vaccine heterologous neutralization tests were performed on paired serum and nasal wash specimens obtained before and one month following vaccination. No significant rises in neutralizing activity were observed in the nasal wash or serum to heterologous rhinovirus serotypes following vaccination with RV13, suggesting that the nasal secretory antibody induced by RV13 vaccine was specific for RV13.

F. Protection From Live Virus Challenge Induced by Intranasal Vaccination Following challenge common cold-like illness developed in 4 of 10 seronegative volunteers but not in any of the 8 intranasal vaccinees who had received vaccine 6 months previously. The difference in occurrence of disease in these two groups was not statistically significantly different because of the low frequency of illness in the seronegative men. Despite our failure to demonstrate an unequivocal effect against disease there was evidence that the intranasal vaccinees were resistant to RV13 infection 6 months post-vaccination because both the duration and the extent of virus shedding was significantly diminished in the vaccine group.

G. Antibody Response of Individuals Who Had Prior Experience with the Antigens of Rhinovirus 13 - A quantity of vaccine was given IN which did not elicit a local or serum antibody response in men with no evidence of previous experience with RV13 antigens; however, this dose of vaccine stimulated serum and/or local antibody response in a proportion of the immunologically experienced men. This result suggests that the local respiratory antibody system possesses some degree of immunologic memory. Previously other investigators had sought such evidence of immunologic memory in the local antibody system without success.

Significance of the Program to the Institute:

With other respiratory tract pathogens, such as RS virus, influenza A virus and *Mycoplasma pneumoniae*, temperature sensitive (ts) mutants have consistently exhibited a pattern of diminished infection in the hamster, a host in which each of these agents replicates. Furthermore, there is a correlation between degree of limitation of infection and the extent of the temperature sensitivity of the mutant. Mutants with the lowest restrictive temperature exhibit the most limited growth in the lower respiratory tract, the region in which disease manifestations are most severe. Temperature sensitivity, i.e., the inability of the mutant to replicate at 37°-39°C, does not necessarily pose an impediment to its capacity to infect and replicate in the upper respiratory passages (nasal turbinates, etc.) since the temperature in this region varies between 32° to 34°C. However, there is also a correlation between defectiveness (i.e., shut-off temperature) of a mutant and capacity to grow in the upper respiratory tract. The experience with the parainfluenza type 1 mutant conforms to these patterns and expectation Temperature limitation of the mutant at 38-39°C was not associated with demonstrable attenuation for the upper respiratory tract of adult volunteers. From these findings it would appear that a type 1 virus mutant of greater defectiveness will probably be required to produce the desired silent type of infection needed for successful immunoprophylaxis.

Although a final influenza vaccine product did not emerge from this study, we have advanced a considerable distance toward this goal. It is clear that ts mutations in influenza virus result in attenuation of virulence for man. The degree of attenuation is related to the degree of temperature sensitivity. The ts-2 recombinant with a shut-off temperature of 39°C is moderately attenuated, while the ts-1 recombinant is over-attenuated in that it does not infect when given at a dosage of 10^5 pfu. Also, we now know that it is possible to attenuate influenza virus by introducing a ts defect into the virus through the mechanism of recombination. Furthermore, infection with an attenuated ts mutant (the Hong Kong ts-2 recombinant) induces significant resistance to challenge with wild type virus. Finally, the ts-2 recombinant is stable in vivo since we did not detect reversion to wild phenotype. What is probably required for immunization against contemporary influenza A viruses is a ts mutant which is intermediate in defectiveness between the ts-1 and ts-2 recombinants, possibly a ts mutant which has a shut-off temperature of 38°C. Once we have identified the proper in vitro laboratory marker, i.e., temperature limitation, it should be possible to update live influenza virus vaccines to meet the threat of new antigenic variants by transferring ts lesions to the new virus through recombination.

Previous studies with the RV13 inactivated vaccine given intranasally have shown that it effectively induced resistance to experimental rhinovirus illness, whereas when administered intramuscularly it was without effect. This experience indicated that local intranasal vaccination was the more effective method for prevention of rhinovirus respiratory tract illness. However, before one can consider local vaccination a practical method, more must be known about the behavior of the local antibody system. Such information has significance for the immunoprophylaxis of rhinovirus disease as well as many other respiratory tract diseases.

Proposed Course of Study:

Work with the cold-adapted type 1 parainfluenza virus mutant will be suspended. Future clinical trials await the development of a ts mutant of type 1 virus with the desired degree of defectiveness.

Attempts to develop a live influenza A virus vaccine will continue and take 3 courses. First, we will evaluate the infectivity of the Hong Kong ts-1 recombinant grown in eggs. This recombinant, which did not infect volunteers at a dosage of 10^5 pfu, can now be grown to a titer of 10^7 pfu per ml in RIF free eggs to which a small quantity of cortisone is added. This higher titered virus suspension may be capable of infecting volunteers. Second, Hong Kong ts-1 recombinants with a shut-off temperature of 38°C will be sought for evaluation in volunteers. Third, ts mutants of Hong Kong virus will be produced directly by chemical mutagenesis with 5 fluorouracil. Mutants with the desired properties will then be studied in man.

Studies are presently underway wherein more sensitive radioimmunologic tests for detecting nasal secretory antibody are being sought so that we may better quantitate the local respiratory tract antibody response. Further studies in volunteers with the RV13 vaccine are underway wherein we will extend our observations on the degree of immunologic memory in the local antibody system. We will also attempt to develop an animal model in which to study nasal secretory antibody production. In this manner it may be possible to study the effect of neonatal thymectomy and the effect of various immunosuppressive agents on nasal antibody production.

Honors and Awards: None

Publications:

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1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Title: Role of Delayed Hypersensitivity in the Immune Response to Respiratory Viral Infections

Previous Serial Number: None

Principal Investigator: Roger E. Wetherbee, M.D.

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Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	15/12			15/12
Professional:	12/12			12/12
Other:	3/12			3/12

Project Description:

Objectives: (1) To study a population of children for (a) incidence of delayed hypersensitivity (D.H.) to parainfluenza 1 and 3, respiratory syncytial virus, and rhinovirus 13; and (b) to determine the correlation between D.H. and presence of serum antibody and nasal secretory IgA to these viruses in this population. (2) To evaluate volunteers participating in rhinovirus vaccine studies for the appearance of D.H. and to characterize this response. (3) To characterize the appearance of D.H. in experimental parainfluenza 3 infection of rabbits and to determine its contribution to the pathophysiology of the pneumonia produced in this system.

Methods Employed:

(1) Assay Systems - A number of *in vitro* correlates of delayed hypersensitivity are known and are useful as assay systems. Among these are migration inhibitory factor (MIF) and lymphotoxin (LT) which are factors released by sensitized lymphocytes after exposure to antigen. These lymphocytes may also dedifferentiate to blast cells with accompanying DNA synthesis or may exhibit a capability for direct cell mediated cytotoxicity.

A. Human direct M.I.F. assay - Surgical specimens of tonsil tissue, transported to NIH in cold Hanks' balanced salt solution are a suitable source of cells for use in a direct, single step MIF assay analogous to the lymph node cell or peritoneal exudate cell model in the guinea pig. This system has the added advantage of making use of lymphoid cells directly contiguous with the upper respiratory tract.

B. Human indirect M.I.F. assay - There is no convenient source of indicator tissue analogous to the tonsil from volunteers participating in routine rhinovirus vaccine studies. Therefore, an indirect, two stage MIF assay is necessary, where lymphocytes from the test individual are obtained from the peripheral blood, incubated for 48 to 72 hours with antigen, and the assay then run using a concentrate of the supernatant incubation fluid and normal guinea pig peritoneal exudate cells as the indicator cells.

C. Rabbit direct M.I.F. assay - Peritoneal exudate cells induced in the rabbit by sterile intraperitoneal oil apparently behave similarly to guinea pig peritoneal exudate cells in a direct indicator system and appear to be a satisfactory source of cells for evaluation of D.H. in the parainfluenza 3 rabbit model.

(2) Serologic Screening Techniques - Routine neutralization and complement fixation tests already well described are used in the screening of nasal washes and serum specimens.

(3) Sources of Test Subjects and Tissues - The population of children being screened for parainfluenza, respiratory syncytial, and rhinovirus is drawn from private patients at Children's Hospital undergoing routine tonsillectomy. The surgical specimens of tonsil tissue are transported to NIH in cold Hanks' balanced salt solution.

The rhinovirus vaccine study volunteers are inmates of Lorton Reformatory, all male and 21 years of age or older. Specimens of heparinized blood are drawn from the volunteers and brought to the NIH for processing.

Major Findings:

A. Feasibility of Direct MIF assay using human tonsil tissue - Although a direct assay system employing human-lymphoid cells offers a clear advantage over the two-stage system using guinea pig indicator cells from a technical viewpoint, either an unacceptably large volume of blood must be taken from each subject or the subject must undergo an operative procedure to procure an appropriate tissue specimen.

Routine tonsillectomy affords a plentiful and uniform supply of viable lymphoid tissue from a population of children from whom a medical history and nasal wash and blood specimens are available. Using established tissue culture techniques,a cell suspension derived from minced tonsillar tissue readily demonstrates an ability to actively migrate over a glass surface which is dose related to the concentration of serum in the medium. In addition, this system displays migration inhibition when exposed to delayed hypersensitivity-evoking antigens such as PPD and streptokinase/streptodornase.

Currently, gradient purified, formalin inactivated whole virus preparations of parainfluenza, respiratory syncytial virus, and rhinovirus are being prepared and standardized for use in this and the indirect assay systems.

B. Feasibility of Rabbit Direct M.I.F. Assay - Preliminary experiments indicate that rabbit peritoneal exudate cells possess the ability to actively migrate over glass in the presence of serum and are inhibited in the presence of antigens to which the animals have been sensitized and which evoke a positive Mantoux-type skin test.

C. Delayed Hypersensitivity After Rhinovirus 13 Vaccination - Six men participating in a rhinovirus vaccine program were examined for serum neutralizing titer and delayed hypersensitivity eight months after receiving an inactivated rhinovirus 13 vaccine intranasally. Three had circulating neutralizing antibody titers of less than 1:2 and three were $\geq$1:32. Two of the men exhibiting low serum titers had failed to respond to the vaccine eight months previously. None demonstrated significant MIF activity *in vitro*.

If there is induction of D.H. during the course of rhinovirus infection, it may be a short-lived phenomenon requiring examination during the acute course of infection and the early convalescent phase. As new vaccine studies are initiated this will be assessed.

Significance of the Program to the Institute:

The contribution of D.H. to the pathophysiology of respiratory viral diseases is essentially unknown but may be significant. The elucidation of this phenomenon will be useful in finding more effective ways of treating such infections and in preparing more effective vaccines having fewer undesirable sequelae.

Proposed Course of Project:

The surveys of children undergoing tonsillectomy and of rhinovirus vaccine study volunteers will continue. As vaccine study groups for parainfluenza and respiratory syncytial viruses are organized, this study will be enlarged to study the participants during the acute and convalescent course.

An attempt will be made to produce and safety test viral antigen preparations to be used in skin testing to provide an _in vivo_ correlate of the _in vitro_ assays.

It appears feasible and technically desirable to design an _in vitro_ assay system to measure lymphotoxin and/or direct cell mediated cytotoxicity activity, using tissue culture cells as the indicator and blood leukocytes as the source of sensitive cells. Such a system may eliminate the need for an indirect MIF assay.

Evaluation of the parainfluenza 3-rabbit model system will continue with emphasis on (1) the role of D.H. in the pathophysiology of the pneumonia and (2) whether there is a partitioning effect of D.H. between local lymphoid cells (pulmonary macrophages) and non-pulmonary cells (spleen, lymph nodes, peritoneal exudate).

Honors and Awards: None

Publications: None

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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
July 1, 1970 - June 30, 1971

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

Several members of the staff of LVD were honored for their scientific accomplishments. Dr. Janet Hartley was a co-recipient, with Dr. Robert Huebner, NCI, of the Kimble Methodology Award. Dr. Hilton Levy received the Superior Service Award, and Drs. Samuel Baron and Wallace Rowe received the Meritorious Service Medal.

The research program of LVD deals chiefly with the biology of oncogenic viruses and with the role of host defense mechanisms, particularly interferon, in determining the course of viral infections. Tumor virus systems under study are murine leukemia virus infection, Marek's disease of chickens, SV40, Adeno-SV40 hybrid viruses, and Visna virus. The interferon program encompasses studies of the biochemical mechanism of action of interferon, and studies of the mechanism, dose-response relations, and anti-tumor and anti-viral activity of interferon inducers.

DNA polymerase and oncogenic potential demonstrated for Visna virus. Visna virus, the cause of a "slow-virus disease" of sheep, has long been known to share certain biologic properties with C-type RNA viruses. It has now been demonstrated that this virus has the most unique feature of the leukemia viruses: an RNA-dependent DNA polymerase in the virion. Electron microscopic studies also showed that Visna virus is very similar morphologically to B- and C-type particles. Because of these findings, attempts were made to transform mouse cells *in vitro*. Morphologically altered cells were produced, and these produced tumors when transplanted into animals. Progressive pneumonia virus of sheep, isolated in Montana, was shown to be closely related to Visna, and to possess the polymerase and ability to transform cells. These studies have profound significance for the biology of this class of slow virus infection. (Takemoto, Stone, Mattern).

Restriction of growth of host range variants of murine leukemia virus (MLV) found to be a multiplicity dependent block. We have shown previously that naturally occurring MLV are of two host-range types, N-tropic and B-tropic. By means of titrations done by determining the number of infectious centers produced as a function of multiplicity of infection, it was shown that restrictive cells are infectable, but the dose-response curves show 2-hit, and in some cases, 3-hit kinetics. As a corollary of this, infectious centers register with equal efficiency on either sensitive or resistant mouse cells. These findings point out the importance of using infected cells, rather than free virus, in attempts to detect virus, and clarify some of the problems of the dynamics of infection with leukemia viruses *in vitro* and *in vivo*. (Hartley, Rowe, Pincus).

Mouse gene system for sensitivity to N- and B-tropic MLV found to be identical to Fv-1 locus. We have elucidated a multiple allelic gene system in mice which determines the sensitivity of their cells to infection with naturally occurring host-range variants of MLV. By studying cells of various inbred, hybrid, and partially congenic mouse strains it was shown that this locus is identical to the Fv-1 locus, known to be a determinant of sensitivity to Friend leukemia virus. This indicates that this locus is a major determinant of viral leukemogenesis by naturally occurring MLV. (Pincus, Rowe; Lilly, Albert Einstein Med. Center).

Rescue of murine sarcoma virus genome from non-producer cells found to be a rapid and efficient process. Non-producer lines of cells transformed by murine sarcoma virus (MSV) produce pseudotype sarcoma virus when the cells are superinfected with MLV. It has not been known whether this was a rare event in a cell population. By means of an inverse infectious center technique where the infectious process is stopped by ultraviolet irradiation, we have found that when the cells are infected with MLV, every cell producing MLV also produces MSV, and both viruses develop simultaneously. This indicates that the MSV is either episomal, or if integrated into cellular nucleic acid, is specifically excised as a result of MLV infection. (Rowe).

New nondefective adeno-SV40 hybrid viruses found to carry different segments of SV40 genome. Five nondefective hybrids have now been isolated and characterized biologically and serologically. In addition, nucleic acid hybridization competition studies have characterized the classes of SV40 RNA synthesized. Each hybrid produces a distinctive spectrum of SV40 RNA, ranging from an amount equivalent to one gene to an equivalent of about four genes. These hybrids thus offer unparalleled tools for relating SV40 gene sequences to biologic functions. (Lewis; Levine, Siegel, NCI; Henry, Samaha, U. of Missouri; Oxman, Levin, Harvard).

Adeno-SV40 hybrid DNA molecule visualized. Heteroduplex molecules formed by melting and reannealing the hybrid and non-hybrid DNA molecules extracted from the defective Ad.7-SV40 hybrid, E46$^+$, were visualized by electron microscopy. The SV40 insertion and a corresponding adenovirus deletion were seen as a doubly looped out region of single stranded DNA. The size and location of the insertion and deletion regions in the hybrid DNA could be accurately determined by this technique. This provides a very powerful and simple means for characterizing and comparing various hybrid viruses. (Kelly; Rose, LBV).

Studies of dosage schedules of interferon inducer leads to improved therapeutic regimen. Because of the hyporesponsive stage which follows induction of interferon by poly I: poly C, extensive studies were done to determine dose schedules to give maximal and prolonged interferon response in mice. By using the optimal dose schedule, significantly improved therapeutic effects were obtained in treating experimental virus infections. In some instances therapeutic effects could be demonstrated when treatment was started quite late in the course of the infection. These studies could be of great significance for adapting poly I: poly C to clinical usage. (Baron, duBuy, Buckler, Worthington).

Messenger RNA patterns altered by interferon treatment. It has been inferred that interferon acts on cells by derepressing a gene for synthesis of an antiviral protein. In studies of mRNA synthesis in interferon-treated cells, a marked change in mRNA pattern was found, with several new species or changed concentrations of regular species of mRNA. Such marked modifications indicate that the cell's response to interferon treatment is more extensive than just the development of one antiviral protein. (Levy).

Antitumor effect of Poly I: Poly C due, at least in part, to activity as an immunologic enhancer. Poly I: poly C has been found to be an effective enhancer of both cell-mediated immunologic activity and humoral antibody production. This finding may explain an important part of the mechanism of the antitumor activity of this drug. (Levy; Asofsky, LMI).

Protozoal infection induces resistance to LDV infection. Infection of mice with either of two protozoa, Leishmania donovani or Plasmodium berghei, produces a marked resistance to infection with the lactic dehydrogenase virus (LDV). Since LDV is thought to replicate only in macrophages, it was postulated that the protozoal infection acted by modifying the macrophages. This was confirmed by the finding that the macrophages from the infected animals were resistant to LDV infection in vitro. (duBuy, Herman, LPD).

Virus infections of Entamoeba histolytica discovered. A number of strains of virus, possibly representing two distinct types of virus, have been found to be indigenous to E. histolytica. They produce lysis of certain strains of the ameba, and can be passaged serially. One virus strain has been visualized, and is polyhedral with a diameter of about 70 mmu. This finding represents the first discovery of a true virus infection of protozoa, and opens a wide field of investigation in a field of great public health importance. (Mattern; Diamond, LPD).

Polypeptide subunits of AAV identified. By acrylamide gel separation of the proteins of purified adenovirus-associated viruses (AAV), three distinct polypeptide subunits have been identified. This development may provide an important step toward determining the molecular basis of their defectiveness, and toward definitive classification of the parvoviruses. (Hoggan, Johnson).

Autoimmune disease in man and mouse. A radioimmunoassay has been developed previously in this laboratory to measure antibodies to DNA in patients with systemic lupus erythematosus. This assay has been applied successfully in diagnosis and management of patients in the pediatric age group and to the study of NZB mice. The immunoglobulin has been characterized as a true antibody to DNA by demonstration of activity in Fab and $F(ab')_2$ fragments. Reaction conditions affecting the interaction of nucleic acids and immunoglobulins have been clarified and the radioimmunoassay has been used by others in this laboratory and elsewhere to measure antibodies to synthetic and natural RNA's as well.

The recent identification of electron microscopic structures as well as serologic studies in patients with systemic lupus have led to the suggestion of a viral etiology of this disease. Studies in this laboratory indicate a generalized hyperreactivity to many viral and non-viral antigens in such patients. The ultrastructures described as "viral particles" do not appear to be viral structures, but are associated with many different viruses, supporting a possible viral etiology, though no specific virus has yet been identified. (Pincus; Blacklow, LID; Kaplan, Steinberg, Talal, NIAMD; Grimley, NCI; Bellanti, Tina, D. Pincus, Georgetown U. Med. Center; Hughes, Columbia Presbyterian Med. Center).

Serial No. NIAID 68H
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunologic and Other Studies of Animal Leukemia Viruses.

Previous Serial Number: SAME.

Principal Investigator: Dr. M.K. Cook.

Other Investigators: Dr. W.P. Rowe, Miss Johnna Sears.

Cooperating Units: Dr. Calvin G. Aulisio and Mr. William Jahnes (DBS); Division of Research Services, L.A.; Dr. J. Newman, Univ. of Md.; Dr. J. Jemski and Mr. E. Tomik, Aerobiology Division, Fort Detrick, Md.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	27/12	3/12	0	30/12
Professional:	24/12	0	0	24/12
Other:	3/12	3/12	0	6/12

Project Description:

Objectives: Study of the biology and natural history of agents associated with Marek's disease and of Type I (RIF-type) and Type III (RE-type) avian leukemia viruses.

Methods Employed: Tissue culture systems and standard animal inoculation procedures were employed. Current methods were used to define and characterize viruses isolated. Immunofluorescence, complement-fixation, agar-gel diffusion and immuno-electrophoresis techniques, and neutralization tests are employed to define similarities and differences in the antigenic components of avian leukosis viruses.

Major Findings: Further and more extensive animal studies employing cell-free chick syncytial virus (CSV), RIF and Marek's disease-free, Marek's disease susceptible chickens and germ-free isolation facilities at Fort Detrick have extended and confirmed an association of CSV and Marek's disease. The highly infectious nature of CSV has been confirmed by the use of control contact birds placed in isolation with chicks inoculated at 1-day of age with CSV. An RNA dependent DNA polymerase was shown associated with CSV.

Clinical signs of Marek's disease were not produced in susceptible isolated chickens inoculated at 1 day of age with cell-free preparations of the GA or JM strains of the herpes type virus associated with Marek's disease (HTV-MD). HTV-MD viremia was demonstrated in most of the birds 30-50 days after inoculation and contact control birds also showed a viremia but histologic examination of the peripheral nerves failed to show Marek's disease lesions in any of the birds.

Early tissue culture passages of field isolates of HTV-MD, in which whole, viable, infected tissue culture cells are used, have produced overt Marek's disease in the birds. Attempts to separate fresh field isolates of HTV-MD from cells have given consistently negative results.

Conventional virus neutralization tests using cell-free HTV-MD indicate that neutralizing antibody can be demonstrated approximately 55 days after inoculation of 1-day-old birds. Agar gel diffusion test results employing JM strain of HTV-MD infected chick embryo fibroblasts or 20X-concentrated supernatant tissue culture fluids, have correlated well with the neutralization test data. The antibody demonstrated in both the agar gel diffusion tests and the neutralization tests appears much later than that shown by the use of immunofluorescence tests where antibody can be demonstrated as early as 12-15 days after infection.

At the present time, the neutralizing and precipitating antibody in HTV-MD infected birds does not appear to have a direct relationship to the ability of the infected animal to recover from viremia or clinical disease.

Sera from Burkitt's lymphoma patients, infectious mononucleosis, and acute leukemia patients, and normal control human sera were all negative for HTV-MD and CSV antibodies. Surveys of poultry handlers and laboratory workers in intimate contact with poultry were made, and antibodies to CSV and HTV-Md were not found.

Proposed Course of the Project: Laboratory studies, including biophysical studies of the cell-associated virus versus extracellular virus and the changes in virus-cell association that lead to the production of extra-cellular virus, will be made. Further attempts will be made to determine if HTV-MD is masking another agent (such as CSV) which may be the actual inducer of lympho-proliferative disease.

The "new-house syndrome," in which Marek's disease develops in chickens reared in new houses with no known contact with diseased birds or with caretakers who may carry the disease, will be studied.

Although the newly licensed Marek's disease vaccine appears to be highly effective in the prevention of clinical Marek's disease, it has been reported that the protection is not based on antibody function or interferon production. A major effort will be made to study the immune phenomenon operating in this disease. The levels and types of globulin in infected birds will be studied.

Differences in cultured macrophages from infected, non-infected, and immune birds will be studied by migration-inhibition and other tests. The presence of a possible chemotoxin-generating factor from infected cells will be studied.

Significance to Biomedical Research and the Program of the Institute: Candidate viruses for the etiologic agent(s) of Marek's disease offer models for the study of lympho-proliferative and tumor viruses in man and animals. It is also important to know the effect of contamination by such agents in viral vaccines prepared in tissue cultures of avian tissue. Mechanisms of cellular immunity studied by using the HTV-MD system may help resolve some problems in tumor regression and hypersensitivity reactions.

Serial No. NIAID 71A
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Parvoviruses with Emphasis on the Adenovirus-Associated Viruses (AAV).

Previous Serial Number: SAME.

Principal Investigators: Dr. M. David Hoggan,
Dr. F. Brent Johnson.

Other Investigators: Dr. Wallace P. Rowe.

Cooperating Units: (1) Dr. Neil R. Blacklow, LID/NIAID (project #661),
Dr. William T. London, PR/NINDS,
Dr. Harvey Ozer, GL&C/NCI,
Dr. Robert Purcell, LID/NIAID.

(2) Dr. Donald W. Roberts, Boyce Thompson Institute for Plant Research, Yonkers, New York; Dr. Max Bergoin, Institut National de la Recherche Agronomique, Station de Recherches Cytopathologiques Republique Francaise.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	20/12	12/12	9/12	41/12
Professional:	20/12	0	9/12	29/12
Others:	0	12/12	0	12/12

Project Description:

Objectives: To study the biological immunological, biophysical, and biochemical properties of the small (less than 30 nm) DNA viruses known as the Parvovirus group. Studies shall continue on their natural history and spread in their natural and incidental hosts in order to investigate their possible role in disease.

To further study their many similar biological and physical characteristics and relate these characteristics to their replication in *in vitro* cell culture systems and to their *in vivo* disease capacities.

Although no disease in humans is yet known for these viruses, it has been shown that AAV-types 1, 2, 3, 4, and H-1 virus can replicate in humans and/or other primates. Further suggestive evidence has been reported that certain epidemics of infectious hepatitis have shown a disproportionate number of antibody rises against AAV-3. Because the Parvoviruses are extremely resistant to inactivation, can remain inactive for months to years in their hosts and on inanimate fomites, and are capable of causing many different types of disease in various hosts, they are prime suspects as the possible etiological agents of numerous chronic diseases.

To develop and study in vivo model systems of in utero infection of AAV in order to investigate the possible role of these agents in perinatal disease.

To continue studies of the Parvovirus proteins, which will provide the basis for additional comparative studies, as well as basic information on the total virus genome.

New Parvovirus candidates will be studied as they are isolated and made available from our own and other laboratories, including porcine Parvoviruses, suspected human hepatitis isolates, bovine adenovirus-associated virus, and certain bacteriophages.

Methods Employed: As pointed out in previous reports, the various Parvoviruses or Parvovirus candidates are purified by various techniques and used as purified antigens for the production of specific antiserum. The purified viruses and respective antiserum are then used for our comparative studies. Examination by electron microscopy and characterization of the various proteins by polyacrylamide gel electrophoresis are carried out on each virus after the viral proteins are broken into their constituent polypeptides after dissociation on sodium dodecyl sulfate and reducing agent. Antiserum is prepared against whole and disrupted virus as well as the various purified protein components.

Serum surveys for AAV-antibodies in various human populations with specific disease syndromes were carried out using purified AAV antigens in the CF test (see project report Blacklow, LID/NIAID, #661).

Because of recent reports that the Detroit-6 cell line was successful in the isolation of some agent associated with MS-1 type infectious hepatitis, which had characteristics of the Parvovirus group, these cells (provided by Dr. Robert Purcell, LID/NIAID) were tested for their susceptibility to various Parvoviruses.

During previous collaborative studies with Dr. William T. London of PR/NINDS, (see 1969-70 Report) it was found that many of the pregnant monkeys in Dr. London's Rhesus breeding colony were free of demonstrable AAV or adenovirus antibody. Since it was known from various serological studies carried out in our laboratory in collaboration with Dr. N. R. Blacklow, that AAV does infect Rhesus monkeys, these findings offered an excellent opportunity to study AAV infection in a primate model system. This seemed important since it was already known that many of the Parvoviruses required actively dividing cells for replication, and some produced spectacular disease in newborn animals or congenital diseases by infection of pregnant mothers. These are exemplified by the findings that cerebellar hypoplasia develops in newborn hamsters or cats infected with rat virus (RV) and in newborn cats infected with feline panleukopenia virus. The disease can also be produced in young cats which are infected in utero with panleukopenia. Further, spontaneous hepatitis and cerebellar hypoplasia are produced in suckling rats by congenital infection.

Rhesus fetuses were infected with AAV-1 in utero in the presence and absence of adenovirus SV-15 as helper at various times after gestation. The mothers were followed closely by daily physical examination, and serological and virological surveillance procedures were carried out at appropriate intervals. If the injected animals did not abort, they were removed by C-section a few days before term. The newborn monkeys were then followed closely by physical, virological, and immunological techniques.

Major Findings: Collaborative studies with Dr. N. R. Blacklow and others on the epidemiology of AAV in humans revealed that AAV-2 and/or AAV-3 infects children primarily during adenovirus infection characterized by severe respiratory disease, including pneumonia, bronchiolitis, croup, and pharyngitis-bronchitis. Few children seroconverted during the summer months of June through September during the 8-year period under study.

Data were also obtained that children with Burkitt's syndrome, known to be etiologically associated with EB virus infection, showed no greater rate of AAV infection than children in the normal population.

In the first series of experiments in the Rhesus monkey model system all mother monkeys receiving either SV-15 plus AAV-1 or SV-15 via the fetus developed antibody against SV-15 and virus was isolated from the mother. On the other hand, only those monkeys receiving AAV-1 plus adenovirus developed antibody to AAV-1. The fetuses surviving who were injected with AAV alone in utero were challenged with SV-15, as were their mothers. They developed antibody to the SV-15, but not to the AAV-1 which was given in utero. Thus, in these cases there was no evidence of AAV-1 remaining in the fetus.

A second group of monkeys has been injected but results are still pending.

Experiments designed to test the susceptibility of the Detroit-6 cell for Parvoviruses revealed that R.V. and Haden virus replicated to a limited extent in these cells, while H-1 virus replicated very well. Of much more interest was the finding that these viruses were helped in these cells by adenovirus and that AAV-1 was maintained in these cultures for at least 14 passages (split 1:400 each time) in the absence of helper adenovirus. It should be pointed out that after passage 8 or 9 adenovirus helper was required to detect the virus.

Antiserum has been prepared against the bacteriophage ØX 174 and a CF test developed because of the similarity of this bacteriophage to the Parvoviruses.

Early studies indicated that besides the two bands of AAV virions found in AAV preparations, consisting of the main infectious band at a density of approximately 1.40 gm/cm and a partially empty band at 1.34-1.37 gm/cm^3, there was frequently a heavy band at 1.45 to 1.47 gm/cm^3. The particles in this band have been found to be infectious and serologically indistinguishable from those in the major band, although they appear to be less antigenic, 12% smaller in diameter and less stable in CsCl. We have now found these dense bands in association with AAV-1, 2, 3, H-1, RV, MVM, and Haden viruses.

Our studies on the proteins have progressed to the point that we can say that the AAV have 3 major proteins with molecular weights of 66,000 ± 2,900 Daltons, 19,700 ± 2,200 Daltons, and 92,200 ± 3,000 Daltons. These proteins are larger than those reported for RV and occur in both the primary upper (1.40 gm/cm^3) band and the lower (1.45 or 1.47 gm/cm^3) band. These proteins are immunogenic in guinea pigs and the lighter protein accounts for more than 80% of the protein of the whole virion.

Significance to Biomedical Research and the Program of the Institute: The stability characteristics of the Parvoviruses as well as their ability to reinfect their specific hosts in the presence of existing antibody make the Parvoviruses or viruses like them possible candidates as etiologic agents for certain chronic and acute diseases suspected of being caused by viruses. These include infectious hepatitis, certain progressive encephalitides, etc.

The fact that all of the Parvoviruses thus far studied in detail have been found to contain single stranded DNA and that many of the hypersensitivity syndromes, including various arthritic conditions, show a propensity for the production of anti-single-stranded DNA antibody formation, needs investigation.

The limited genetic information of 1.5-1.7 X 10^6 Daltons of DNA continues to make these agents ideal for the detailed study of virus replication at the molecular level.

Proposed Course of Project: The comparative studies will continue and clues as to their possible role in disease will be watched for as the number and variety of these agents continue to increase. These viruses continue to be outstanding candidates for widening our basic knowledge of virus replication and host's synthetic processes. Studies to elucidate their replicative cycles and possible genetic derivation will be pursued.

Invited Seminars and Papers:

1. 10th International Congress for Microbiology, Mexico City (Aug. 1970). Round Table - Comparative, morphological, chemical, and immunological studies of small DNA viruses.

2. Second International Congress for Virology, Budapest, Hungary (June 1971). Co-chairman Workshop in Small Isometric DNA Viruses.

Publications:

Hoggan, M.D.: Small DNA viruses. In: Maramorosch, I. and Kurstak, E. (Eds.). Comparative Virology, pp. 43-79, Academic Press, New York, New York (1971).

Blacklow, N.R., Hoggan, M.D., and McClanaham, M.S.: Adenovirus-associated viruses: enhancement by human herpesviruses. Proc. Soc. Exp. Biol. Med., 134: 952-954 (1970).

Blacklow, N.R., Dolin, R., and Hoggan, M.D.: Studies of the enhancement of an adenovirus-associated virus by herpes simplex virus. J. Gen. Virol., 10: 29-36 (1971).

Blacklow, N.R., Hoggan, M.D., Sereno, M.S., Brandt, C.P., Kim, H.W., Parrot, R.H., and Chanock, R.M.: A seroepidemiologic study of adenovirus-associated virus infection in infants and children. Am. J. Epid. (in press).

Serial No. NIAID 71B
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Indigenous Viruses of Mice and Other Species with Special Reference to their Importance as Extraneous Viruses in Mouse Tumor Systems.

Previous Serial Number: SAME.

Principal Investigators: Drs. Wallace P. Rowe and Theodore Pincus.

Other Investigators: None.

Cooperating Units: Dr. Neil R. Blacklow, LID/NIAID.
Dr. N. Talal, A&R/NIAMD
Dr. P. Grimley, LP/NCI
Dr. J. Bellanti, Georgetown University School of Medicine, Washington, D.C.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2/12	2/12	2/12	6/12
Professional:	1/12	2/12	0	3/12
Other:	1/12	0	2/12	3/12

Project Description:

Objectives: To study the properties and natural history of viruses of laboratory animals and man, with particular regard to their effects on tumor virus study systems and the biology of cancer.

Methods Employed: Standard virologic and epidemiologic procedures for detection and characterization of viruses, using serologic, tissue culture, and animal tests; electron microscopy; radioisotope precipitation test for antibodies to nucleic acids.

Major Findings:

Cytoxan protection against LCM: We reported in 1954 that induction of lymphopenia protected mice against the lethality of LCM virus infection. We recently studied the effect of timing of cytoxan treatment on this sparing phenomenon, and found an interesting paradoxical effect. Treatment during the first half of the incubation period protected against death caused by large doses of virus, but not against that caused by small doses. Treatment during the latter half of the incubation period protected against all doses. Growth of virus was not affected by the treatment. We interpret these results to indicate that the lymphopenia must be present at the end of the usual latent period in order to prevent the lethal inflammatory response.

Autoimmune disease in man and mouse. A radioimmunoassay has been developed previously in this laboratory to measure antibodies to DNA in patients with systemic lupus erythematosus. This assay has been applied successfully in diagnosis and management of patients in the pediatric age group and to the study of NZB mice. The immunoglobulin has been characterized as a true antibody to DNA by demonstration of activity in Fab and $F(ab')_2$ fragments.

Reaction conditions affecting the interaction of nucleic acids and immunoglobulins have been clarified and the radioimmunoassay has been used by others in this laboratory and elsewhere to measure antibodies to synthetic and natural RNA's as well.

The recent identification of electron microscopic structures as well as serologic studies in patients with systemic lupus have led to the suggestion of a viral etiology of this disease. Studies in this laboratory indicate a generalized hyperreactivity to many viral and non-viral antigens in such patients. The ultrastructures described as "viral particles" do not appear to be viral structures, but are associated with many different viruses, supporting a possible viral etiology, though no specific virus has yet been identified.

Significance to Biomedical Research and the Program of the Institute:

Virus infections of laboratory animals are a major problem in experimental work, both because of their effects on health of the animals and by contaminating experimental materials. Our continuing program for identification and detection of indigenous rodent viruses has led to great improvement in the quality of laboratory mice and in the prevention and control of viral contamination of experimental systems.

The radioisotope precipitation test for antibodies to DNA and RNA is a procedure of great importance in clinical diagnosis of autoimmune disease and in experimental studies of autoimmune disease and the immunologic response to nucleic acids.

Proposed Course:

Studies of LCM virus infection in tissue culture will continue, with particular reference to the self-sterilizing system which we have developed. Studies of indigenous mouse viruses will be undertaken only as the need arises.

Publications:

Pincus, T. and Kaplan, A.P.: Evidence for true antibodies to DNA in systemic lupus erythematosus: Activity of Fab and $F(ab')_2$ fragments. Nature, 227: 394, 1970.

Pincus, T., Blacklow, N., Grimley, P., and Bellanti, J.: Glomerular microtubules of systemic lupus erythematosus. The Lancet, 1058-1061, 1970.

Pincus, T.: Immunochemical studies of DNA antibodies using ammonium sulfate presentation. Arthritis and Rheumatism, in press.

Steinberg, A.D., Pincus, T., and Talal, N.: The pathogenesis of autoimmunity in New Zealand mice. III. Factors influencing the formation of anti-nucleic acid antibodies. Immunology, in press.

Pincus, T., Hughes, G.R.V., Pincus, D., Tina, L., and Bellanti, J.: DNA antibodies in childhood systemic lupus erythematosus. J. of Pediatrics, in press.

Serial Number NIAID/NCI 71 D
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Project Title: Studies of Mouse Leukemia Viruses.

Previous Serial Number: SAME

Principal Investigators: Janet W. Hartley, Ph.D. (LVD,NIAID)
Wallace P. Rowe, M.D. (LVD,NIAID)
Robert J. Huebner, M.D. (VCB,NCI)

Other Investigators: Natalie M. Teich, Ph.D. (LVD,NIAID)
Douglas R. Lowy, M.D. (LVD,NIAID)
Theodore Pincus, M.D. (LP,NCI)

Cooperating Units: Dr. Stuart Aaronson (VCB,NCI)
Dr. Aaron Freeman (Microbiological Associates)
Dr. Roger E. Wilsnack (Huntingdon Laboratories)
Dr. Frank Lilly (Albert Einstein College of Medicine, New York)

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	83/12	42/12	12/12	137/12
Professional:	64/12	27/12	12/12	103/12
Others:	19/12	15/12	0	34/12

Project Description:

Objectives: To utilize sensitive virus isolation and quantitation procedures for murine leukemia and sarcoma viruses in studying the natural history of infection with these agents and the biology of their growth in tissue culture.

Methods Employed: Tissue culture procedures, using plaque and focus assays and complement fixing antigen induction; complement fixation; fluorescent antibody techniques; tumor induction in mice, hamsters and rats.

Major Findings:

Host range studies: Previous studies have shown that naturally-occurring murine leukemia viruses (MLV) initiate infection significantly more efficiently on embryo cells of either NIH Swiss (N-tropic viruses) or BALB/c (B-tropic viruses) mice. Characterization of the sensitivity of embryo cells of 21 other strains of mice to N- and B-tropic MLV's has indicated that all strains tested were similar either to NIH (N-type) or to BALB/c (B-type). Patterns of sensitivity of N-type X B-type F_1 hybrids indicated dominance of resistance to both types of virus, and the sensitivity of cells from individual backcross embryos indicated that a single locus (N-B) is the primary determinant of host range patterns. Further, in collaborative studies with Dr. Lilly, strong evidence was found for the identity of the N-B locus to the FV-1 locus described for sensitivity to Friend leukemia virus.

The N-B locus does not affect the growth of the laboratory passaged MLV's (Moloney, Rauscher, et al.) which propagated equally on embryo cells of all strains as well as F_1 hybrids; however, certain "NB-tropic" variants derived by passage of B-tropic viruses in NIH cells show a markedly decreased ability to infect DBA (N-type) cells and may provide an indicator for delineation of another allele.

The virology of the observed patterns of virus tropism and genetically determined cell susceptibility has been studied intensively, utilizing the quantitative facility provided by the XC plaque test and infectious center procedures based thereon, which permit the study of individual infected cells. Comparison of the characteristics of infection of sensitive and resistant (actually semi-permissive) cells indicated no significant differences in rate or degree of absorption and penetration, and the yields of virus from an infected sensitive or resistant cell are comparable. However, it appears that the resistant cell requires infection by two (or, in some cases, three) virus particles to become productively infected. Multiplicity effects are also seen in sensitive cells, since productive infections early in the latent period are multi-hit events.

Irradiation studies: Studies of the UV inactivation curves of various parameters of MLV infection have shown three classes of sensitivity. Ability of cells to divide, or to be infected with MLV is extremely sensitive. Ability of infected cells to continue virus production is moderately sensitive; while infectivity of free virus, infectivity of virus-covered cells, and ability of infected cells to induce syncytium formation in XC cells is relatively resistant. This dissociation of functions by UV irradiation provides powerful means for studying the infectious process. For example, infection can be halted during the latent period without destroying the infectivity of those cells that have already started virus production. Also, the ability to kill cells without disturbing their ability to affect XC cells forms the basis of the plaque test for MLV infectivity.

Murine sarcoma virus (MSV): The rescue of the defective sarcoma genome from an MSV non-producer mouse cell line was studied quantitatively. It was found that rescue occurred with high efficiency; all cells producing MLV also produced MSV and the latent period of both viruses in individual cells appeared to be identical.

Serology of MLV's: Continuing studies of the serologic character of naturally-occurring MLV's suggest that isolates are divisible into two broad subgroups--"Gross-type" and "AKR-type." The former subgroup comprises Gross Passage A virus and virus strains neutralizable by Gross Passage A antiserum but not by AKR virus antiserum. AKR-type viruses are those efficiently neutralized by AKR antiserum as well as by Gross antiserum.

Virology of the AKR mouse: Of several cell lines established from cultures initiated with small numbers of AKR embryo cells, two lines were obtained which were free of infectious MLV and MLV-related antigens. Various procedures designed to induce MLV production in these cells are being tested with the aim of obtaining a predictable procedure which could allow quantitative studies of the state of the genome and the processes which allow its expression in such cells.

Quantitative studies of the amount of infectious virus in AKR mice of various ages indicate a changing organ distribution of highest titer virus with increasing age, as well as increasing virus titer in the most active sites with age.

MLV isolates from wild mice: In collaboration with Dr. Freeman, two isolates of infectious virus have been made from embryo cell cultures of rural feral mice. Studies are in progress to confirm the source of virus and study the characteristics of these isolates.

Significance to Biomedical Research and the Program of the Institute:

The biology of murine leukemia virus infection is one of the most important and controversial areas of modern biology; it is also one of the most complex and difficult. There is much evidence indicating that the genetic material of these viruses is transmitted as a genetic or epigenetic element in the germ cells, and that the expression of the viral genome is controlled by host genetic factors. The viral genome can become expressed as a result of aging, carcinogens, and possibly from dedifferentiation of cells. The implications of this model for carcinogenesis, differentiation, immunology, and genetics are immense.

Our laboratory has long considered that investment of its effort into developing meaningful techniques for study of MLV would eventually provide the greatest contribution to this problem. Having now developed sensitive procedures for detecting infectivity and antigens of many of the naturally-occurring MLV's, we feel we have accomplished a large part of this objective.

Proposed Course:

With techniques for rapidly isolating, quantitating, and characterizing MLV's now established, a number of studies related to the biology of MLV infection in various strains of mice are in progress to determine the patterns of virus growth and control in relation to spontaneous or irradiation-induced leukemia. Further, the genetic factors affecting expression and distribution of MLV will be studied in hybrids of mice of different N-B type, with different H_2 alleles, and with differing frequencies of spontaneous leukemia.

Efforts will continue in the study of genome expression in the AKR non-producer cells as well as in other mouse cell lines.

Studies on the factors influencing cell sensitivity to MLV infection will continue.

Honors and Awards:

Dr. Janet W. Hartley: Invited presentation, 10th International Cancer Congress, Houston, Texas, May 1970.

Corecipient, Kimble Methodology Award, October 1970.

Publications:

Aaronson, S.A. and Rowe, W.P.: Nonproducer clones of murine sarcoma virus transformed BALB/3T3 cells. Virology, 42: 9-19, 1970.

Rowe, W.P., Pugh, W.E., and Hartley, J.W.: Plaque assay techniques for murine leukemia viruses. Virology, 42: 1136-1139, 1970.

Pincus, T., Hartley, J.W., and Rowe, W.P.: A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture studies of naturally-occurring viruses. J. Exptl. Med., in press.

Pincus, T., Rowe, W.P., and Lilly, F.: A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to Friend murine leukemia virus. J. Exptl. Med., in press.

Hartley, J.W., Rowe, W.P., and Wilsnack, R.E.: Detection of murine leukemia virus antigens. Proceedings, Tenth International Cancer Congress, May 24, 1970, Houston, Texas, in press.

Levy, J.A. and Pincus, T.: Demonstration of biological activity of a murine leukemia virus of NZB mice. Science, 170: 326-327, 1970.

Serial No. NIAID 71E
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Viral Antigens in Virus-induced Tumors.

Previous Serial Number: SAME.

Principal Investigators: Drs. Andrew M. Lewis, Jr. and Wallace P. Rowe.

Other Investigators: Dr. Thomas J. Kelly.

Cooperating Units: Dr. James A. Rose (LBV,NIAID)
Drs. Arthur Levine and Stuart Siegel (Medicine Branch, NCI), Michael Oxman, Myron Levin (Harvard Medical School), Patrick Henry, Richard Samaha (University of Missouri School of Medicine).

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	49/12	12/12	0	61/12
Professional:	25/12	0	0	25/12
Other:	24/12	12/12	0	36/12

Project Description:

Objectives: To use the adenovirus-SV40 hybrid viruses as tools to study the genetics of DNA tumor viruses; to define the role of viral genes and antigens in the oncogenic process; to study the biology of adenovirus-SV40 hybrid viruses.

Methods Employed: Tissue culture, fluorescent antibody, complement fixation, serologic epidemiology, and nucleic acid homology techniques.

Major Findings: Adenovirus-SV40 hybrid viruses. Several years ago we isolated and began to characterize the first nondefective adenovirus-SV40 hybrid. This virus, designated Ad.$2^{+}ND_1$, contains about 250,000 daltons of SV40 DNA covalently linked to what appears to be the entire Ad.-2 genome. As this hybrid is nondefective, it replicates without helper in both human and monkey kidney cells. It induces an early SV40 protein, the SV40 U antigen which is heat stable and serologically different from the heat labile SV40 T antigen. More recent studies have shown that the SV40 specific RNA species produced by the Ad.$2^{+}ND_1$ virus represent only a portion of the RNA species produced by SV40 early in its replicative cycle prior to synthesis of viral DNA.

The Ad.2^+ND_1 hybrid may then be considered an independently-replicating deletion mutant of SV40. The usefulness of such a mutant in the genetic analysis of SV40 has been demonstrated by the identification of the SV40 U antigen and its association with a small segment of SV40 DNA and a particular species of early SV40 RNA. Isolation and examination of different nondefective Ad.-SV40 hybrids should permit a more detailed analysis of the SV40 genome and allow a correlation of the biologic functions of SV40 with various segments of SV40 DNA and species of SV40 RNA.

More recently we have isolated and begun to characterize 4 additional nondefective adenovirus-SV40 hybrid viruses. These new agents have been designated Ad.2^+ND_2, Ad.2^+ND_{3A}, Ad.2^+ND_{3B}, and Ad.2^+ND_4. They differ from each other and from the original nondefective hybrid Ad.2^+ND_1 in their ability to induce SV40 antigens to plaque and pass serially in African green monkey kidney (AGMK) cells, and to induce certain species of SV40 RNA.

The Ad.2^+ND_2 virus plaques efficiently by 1-hit kinetics in both human embryonic kidney (HEK) and AGMK cells. It does not induce SV40 T or V antigen but does induce SV40 U antigen which is detectable by both complement fixation and immunofluorescence with T^+U^+ hamster serum. In contrast to the perinuclear distribution of SV40 U antigen induced in cells infected with Ad.2^+ND_1, the SV40 U antigen induced by Ad.2^+ND_2 is present throughout the cytoplasm of infected cells. By hybridization-competition, the SV40 RNA induced Ad.2^+ND_1 competes with about 40% of the SV40 RNA induced by Ad.2^+ND_2. Thus this virus induces several more species of SV40 RNA than does the Ad.2^+ND_1 hybrid.

The Ad.2^+ND_{3A} hybrid plaques with 1-hit kinetics in HEK cells. It does not pass serially in AGMK cells and induces no plaques in these cells at 1-10,000 dilutions. No serologically detectable SV40 antigens are present in cells acutely infected with Ad.2^+ND_{3A}. This hybrid does induce small amounts of SV40 specific RNA in infected cells. This RNA competes with approximately 40% of the SV40 RNA sequences present in cells infected with Ad.2^+ND_1.

The Ad.2^+ND_{3B} hybrid is similar to the Ad.2^+ND_{3A} virus in that it plaques only in HEK cells and fails to induce detectable SV40 antigens. However, the SV40 specific RNA species induced by Ad.2^+ND_{3B} compete completely with both Ad.2^+ND_1 and Ad.2^+ND_{3A} and with 80% of the SV40 RNA species induced by the Ad.2^+ND_2 hybrid. Thus it is inducing more species of SV40 RNA than either Ad.2^+ND_1 or Ad.2^+ND_{3A} even though there are no SV40 specific functions associated with these RNA's.

The Ad.2^+ND_4 hybrid plaques with 1-hit kinetics in both HEK and AGMK cells and induces both SV40 T and U antigens. By immunofluorescence only intranuclear staining is detected in cells infected with Ad.2^+ND_4. Thus the Ad.2^+ND_4 hybrid is similar to wild type SV40 and unlike Ad.2^+ND_1 and Ad.2^+ND_2 in that the SV40 U antigen it induces is detectable in the nucleus of infected cells. The SV40 specific RNA sequences induced by Ad.2^+ND_4 compete completely with all the SV40 RNA sequences induced by the other nondefective hybrids.

It has been found that electron-microscopic mapping techniques can be used to determine the detailed structure of the genomes of adenovirus-SV40 hybrids. The first hybrid analyzed by these techniques was E46$^+$PL1, a defective Ad.7-SV40 hybrid. The structure of the genome of this hybrid may be summarized as follows: 1. The hybrid genome contains 16% less Ad.7 DNA than the non-hybrid Ad.7 genome. 2. This deletion consists of the segment of DNA mapping between 0.05 and 0.21 molecular lengths in the non-hybrid Ad.7 DNA molecule. 3. The deleted DNA has been partially replaced by an amount of heterologous DNA equivalent to 75% of the complete SV40 genome. The E46$^+$PL1 genome appears to have arisen via two recombination events between Ad.7 and SV40.

Significance to Biomedical Research and the Program of the Institute: The nondefective adenovirus-SV40 hybrids represent independently replicating deletion mutants of SV40. As such, they offer a unique approach to studying the genetics of a DNA tumor virus. By isolating and characterizing a number of different hybrids we should be able to associate specific SV40 functions and species of RNA with segments of SV40 DNA, and to identify which, if any, SV40 DNA sequences are responsible for its oncogenicity. The isolation of 5 different nondefective hybrids suggests that recombination between the genomes of different animal viruses may be a more frequent event than previously suspected. Similar events occurring in dually infected hosts or humans could explain some of the antigenic variations in animal viruses. The presence of such recombinants among other animal viruses would be very useful for genetic studies.

Proposed Course of the Project: Studies are under way to locate and determine the amount of SV40 DNA within each hybrid and to compare the SV40 RNA species with those species of RNA produced by SV40 during acute infection. Work is also under way to identify which of these agents have the ability to induce other SV40 specific functions such as transplantation rejection antigens, enhancement of human adenovirus replication in AGMK cells and transformation of various cells.

Invited Addresses:

Thomas J. Kelly, Jr. - 1. Johns Hopkins University
2. Carnegie Institution of Washington

Publications:

Crumpacker, C.S., Levin, M.J., Wiese, W.H., Lewis, A.M., Jr., and Rowe, W.P.: Adenovirus type 2-Simian Virus 40 hybrid population: Evidence for a hybrid deoxyribonucleic acid molecule and the absence of adenovirus encapsidated circular Simian Virus 40 deoxyribonucleic acid. J. Virol., 6: 788-794, 1970.

Lewis, A.M., Jr., and Rowe, W.P.: Studies of nondefective Adenovirus-Simian Virus 40 hybrid viruses. I. A newly characterized Simian Virus 40 antigen induced by the Ad.2$^+$ND$_1$ virus. J. Virol., 7: 189-198, 1971.

Levin, M.J., Crumpacker, C.S., Lewis, A.M., Jr., Oxman, M.N., Henry, P.H., and Rowe, W.P.: Studies of nondefective Adenovirus 2-Simian Virus 40 hybrid viruses. II. Relationship of Adenovirus 2 deoxyribonucleic acid and Simian Virus 40 deoxyribonucleic acid in the Ad.2^+ND_1 genome. J. Virol., 7: 343-352, 1971.

Crumpacker, C.S., Henry, P.H., Kakefuda, T., Rowe, W.P., Levin, M.J., and Lewis, A.M., Jr.: Studies of nondefective Adenovirus 2-Simian virus 40 hybrid viruses. III. Base composition, molecular weight, and conformation of the Ad.2^+ND_1 genome. J. Virol., 7: 352-359, 1971.

Kelly, T.J., Jr. and Rose, J.A.: SV40 integration site in an Adenovirus 7-SV40 hybrid DNA molecule. Proc. Nat. Acad. Sci., in press.

Serial No. NIAID-71-F
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Interferon and Interferon Inducers

Previous Serial Number: SAME

Principal Investigator: Dr. Hilton B. Levy

Other Investigators: Dr. Sam Margolis, LVD, NIAID
Mr. Freddie Riley, LVD, NIAID

Cooperating Units: Dr. Werner Braun, Rutgers University, New Jersey
Dr. T. Sreevalsen, Georgetown University, Washington, D. C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3 1/2			3 1/2
Professional	2 1/2			2 1/2
Other	1			1

Project Description:

Objectives

To study the molecular and physiological mechanism of the antiviral and antitumor action of polyinosinic.polycytidylic acid and interferon. To participate in obtaining best conditions for testing interferon inducers in man.

Methods Employed

Many biochemical methods have been employed. Included, but not all inclusive, are spectrophotometry, single and double isotope tracing, gel electrophoresis, gradient centrifugation, paper and column chromatography, etc. In addition, various biological techniques have been employed, both in tissue culture and in animals, as indicated in the sections on results. Several extensions of the intramural program have been developed in collaborative programs through the contract branch of NIAID.

Major Findings

1. Studies on the effects of poly I.poly C (PIC) on macromolecule synthesis in animals and in tissue culture have been completed. In tissue culture cells that are induced to a virus resistant state by PIC, the compound causes an increase in the rate of incorporation of radioactive amino acids into proteins. The macromolecular change is associated with the development of increased permeability of the cells to externally applied radioactive amino acids, and a consequent increase in the radioactivity of the intracellular pool of amino acids. This latter increase is directly responsible for the increased radioactivity in the protein. The development of the increased permeability induced by PIC, on the other hand, requires the synthesis of cellular RNA and protein, suggesting that PIC treatment leads to a modification in cell messenger RNA and protein synthesis; that is--the drug leads to a derepression of one or more cistrons in inducing interferon and resistance to virus. This increased permeability is selective in that permeability to non-metabolized substrates such as methylglucose is not altered.

It was reported in a previous annual report that PIC inhibits macromolecule synthesis in tumors in mice more than it inhibits such synthesis in normal tissues. However, we have found that after repeated injections of PIC a degree of resistance develops and the level of inhibition of macromolecule synthesis decreased. Polysome and messenger RNA profiles are strongly affected in animals receiving PIC.

2. Contract efforts with Dr. T. Sreevalsen of Georgetown University extended our earlier observations on formulation methods of PIC indicating that changes in salt concentration and temperature of annealing can strongly affect the quantitative effectiveness of the drug. This information may be valuable in preparing material for clinical trials.

3. Treatment with interferon of animals with tumors leads to a very marked inhibition of the tumor growth and a prolongation of survival time of the animals. While such

effects are not so strong as those brought about by PIC it might be that larger quantities of interferon than are currently available would be more effective. Further the hydrolytic action against PIC shown by a number of animal sera would not be a problem if interferon itself were used for the therapy.

4. The explanation for the antitumor effect of interferon may come from experiments done in our laboratory and in collaboration with Dr. Werner Braun of Rutgers in which we showed that interferon is an effective enhancer of both cell-mediated immunological activity and humoral antibody production.

5. The presence in man and in a number of species of animals of an enzyme that hydrolyzes double-stranded RNA may limit the usefulness of PIC in its present form. Animals, including man, with a high hydrolytic capacity for PIC respond poorly to the compound in producing interferon. Several of our early attempts to stabilize PIC against this destructive action have not been successful. Collaborative efforts through contracts are planned to extend this approach.

6. Electrophoretic studies on patterns of messenger RNA isolated from polysomes of cells treated with interferon and with PIC reveal the following. Interferon leads to an increase in the amount of messenger RNA produced as well as to the production of new components demonstrable as new peaks in polyacrylamide gel electrophoresis. The shape of these peaks suggest that they represent several new species rather than one. PIC treatment of cells can lead to an overall decrease or increase in messenger RNA synthesis (depending on the concentration of PIC). However, even when the overall RNA synthesis is decreased new species of RNA are detectable.

7. Previous studies from these laboratories showed that the molecular basis of the action of interferon resides in modified ribosomes or ribosome associatesd factors induced by the interferon treatment. These modified ribosomes bind to and translate normal cell messenger RNA as well as do control ribosomes, but the ribosomes from the interferon treated cells bind and translate viral RNA poorly. We also showed that the first interaction of a radioactive RNA virus with a normal cell resulted in the incorporation of the viral RNA into a particle sedimenting at 50S in sucrose gradients. This early association did not occur in cells treated with interferon. We have begun attempts to study the nature of this 50S particle. It has a buoyant density of about 1.50 to 1.52 in cesium chloride. Further analysis of this bouyant density should enable us to determine whether the viral RNA is associated with a ribosomal subunit or with a non-nucleic acid containing cellular component such as a protein.

Significance to Biomedical Research and the Program of the Institute

Results emanating from this project might be considered in two categories; that which is directly related to disease control and that related to basic studies (a) of the nature of cell processes in normal and virus infected hosts and (b) of the defenses against virus infection. At the present time the most promising approach to control of virus disease is through the interferon system. The goal-oriented aspects of this project are directed to bring the interferon system to the stage of clinical usefulness. Along these lines development of better interferon inducers in man is directly relevant.

For the longer range viewpoint the basic studies should lead to further understanding of processes in the cell that control the specificity of protein synthesis by means of alterations in ribosomes and ribosome associated factors. The emergence of a close association between the interferon system and the immunological system is of particular relevance to this institute.

Proposed Course of the Project

The program with poly I.poly C as an antiviral and antitumor agent will to a large extent continue as collaborative efforts through the contract mechanism. These programs will emphasize the development of RNA inducers of interferon that are resistant to the hydrolytic action of human serum, as well as the production of large quantities of human interferon.

For the most part the intramural efforts of the laboratory will be dealing with problems relating to the growth of viruses and to the action of interferon. The nature of the first interaction product between the genome of an infecting virus and constituents of the host cell will be studied. This is particularly important today because of the developments of the past two years showing that there are cytoplasmic factors in cells that become ribosome associated and then control the specificity of translation of RNAs into protein. It is in this area that we feel that interferon asserts its antiviral effect and we plan to do further studies with interferon along these lines.

Honors and Awards:

Organizing Committee -- International Congress on Interferon, Leuven, Belgium, 1970.

Invited Symposium speaker -- International Congress on Virology, Budapest, 1970.

Publications:

Levy, H. B.: The antitumor activity of polyinosinic.polycytidylic acid. In Nagano, Y., and Levy, H. B. (Eds.): Interferon. Tokyo, Igaku Shoin Ltd., 1970, pp. 194-202.

Levy, H. B., Asofsky, R., Riley, F., Garapin, A., Cantor, H., and Adamson, R.: The mechanism of the antitumor action of poly I.poly C. Ann. N. Y. Acad. Sci. 173: 640-648, 1970.

Levy, H. B., Riley, F., and Margolis, S.: Interferon and interferon inducers in the treatment of malignancies. J. Gen. Physiol. 56: 238-248, 1970.

Levy, H. B.: Interferon and interferon inducers in the treatment of malignancies. Arch. Intern. Med. 126: 78-83, 1970.

Levy, H. B.: Genome function and Disease. In Dunlop, R. H., and Moon, H. W. (Eds.): Resistance to Infectious Disease. Saskatoon, Saskatoon Modern Press, 1970, pp. 13-24.

Margolis, S. A., and Levy, H. B.: The action of poly In.Cn on the RNA metabolism of cultured cells. Ann. N. Y. Acad. Sci. 173: 339-345, 1970.

Devita, V. T., Canellos, G. P., Carbone, P. P., Baron, S., Levy, H., and Gralnick, H.: Clinical trails with interferon inducer polyinosinic.polycytidylic acid. In Proc. Amer. Assoc. Cancer Res., 1970, in press.

Hill, D. A., Baron, S., Levy, H. B., Bellanti, J., Buckler, C. E., Cannellos, G., Carbone, P., Chanock, R. M., DeVita, V., Guggenheim, M. A., Homan, E., Kapikian, A. Z., Kirschstein, R. L., Mills, J., Perkins, J. C., VanKirk, J. E., and Worthington, M.: Clinical studies of induction of interferon by polyinosinic.polycytidylic acid. Perspectives in Virology, 1970, in press.

Cantor, H., Asofsky, R., and Levy, H. B.: The effect of polyinosinic.polycytidylic acid upon graft-vs-host activity in balb/c mice. J. Immunol. 104: 1035-1038, 1970.

Levy, H. B., and Riley, F.: The effect of polyinosinic.polycytidylic acid on tumor metabolism. Proc. Soc. Exp. Biol. Med. 135: 141-145, 1970.

Dianzani, F., Baron, S., Levy, H. B., and Buckler, C. E. Mechanisms of enhancement of polynucleotide induction of interferon. In Perkins, F. T. and Regamey, R. H. (Eds.): *Immunobiological Standardization*: International Symposium on Interferon and Interferon Inducers. New York, Karger, Basel, 1970, vol. 14, pp. 203-208.

Baron, S., Bogomolova, N. N., Billiau, A., Levy, H. B., Buckler, C. E., Stern, R., and Naylor, R.: Induction of interferon by preparations of synthetic "single-stranded" RNA. In Chany, C., and DeSomer, P. (Eds.): *L'Interferon* (No. 6). Paris, Institut National De La Sante Et De La Recherche Medicale, 1970, pp. 9-27.

Levy, H. B.: Some problems associated with studying the antitumor activity of polyinosinic.polycytidylic acid. In Perkins, F. T. and Regamey, R. H. (Eds.): *Immunobiological Standardization*: International Symposium on Interferon and Interferon Inducers. New York, Karger, Basel, 1970, vol. 14, pp. 195-198.

Levy, H. B., Law, L. W., Rabson, A. S., Riley, F., Margolis, S., and Asofsky, R.: The effect of polyinosinic. polycytidylic acid on tumor growth. In Chany, C., and DeSomer, P. (Eds.): *L'Interferon* (No. 6). Paris, Institut National De La Sante Et De La Recherche Medicale, 1970, pp. 189-198.

Nagano, Y., and Levy, H. B. (Eds.): *Interferon*. Tokyo, Igaku Shoin Ltd., 1970, 218 pp.

Weinstein, A. B., Gazdar, A. F., Sims, H. L., and Levy, H. B.: Poly I.poly C; Lack of correlation between interferon production and antitumor effect. *Nature*, 1971, in press.

Adamson, R. H., and Levy, H. B.: The interferon system. In Rubin, A. A. (Ed.): *Search for New Drugs* 1971, in press.

Levy, H. B., and Merigan, T. C.: Preparation and mode of action of interferon. *Methods in Molecular Biology*, 1971, in press.

Serial No. NIAID-71-G
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure and Replication of Viruses

Previous Serial Number: SAME

Principal Investigator: Dr. C. F. T. Mattern

Other Investigator: Mr. Wendell A. Daniel

Cooperating Units: Dr. K. K. Takemoto, LVD, NIAID, 71-J
Dr. Louis S. Diamond, LPD, NIAID, 122-G1
Dr. H. G. duBuy, LVD, NIAID, 71-I

Man Years:

	NIAID Lab. Staff	Other NIH Personnel to Project	Guest Workers	TOTAL
Total	1 2/12	1/12		1 3/12
Professional	1	1/12		1 1/12
Other	2/12			2/12

Project Description:

Objectives

The major emphasis on virus structure and development is centered about two main areas. (1) Slow viruses, such as sheep pneumonitis virus and Visna virus are being studied in tissue culture systems. Another slow virus project involves an attempt to identify the causative agent of scrapie in mice. (2) The identification and study of the first known protozoal virus system.

Methods Employed:

These studies involve high resolution electron microscopy of viruses and sections of infected and normal cells employing a variety of stains.

Major Findings

1. Morphogenetic similarities between the sheep pneumonitis virus, a "slow" virus of sheep and Visna virus have been studied and published in collaboration with Dr. K. K. Takemoto and others.

2. A new development in virology has been the discovery with Dr. L. S. Diamond, LPD, of viral agents indigenous to *Entamoeba histolytica*. These agents fulfill the following criteria of viruses. They pass 0.2u filters and can be serially passaged in normal amoeba causing lysis in about 95% of cells in each successive passage. One strain has been passaged more than 30 times at 1:7.5 dilution representing $>1/7.5^{30}$ dilution, thus establishing it as a replicating agent. Particles associated with cells lysed by one virus are polyhedral and are about 70 mu in diameter and resemble well established viral agents. The morphogenesis of two strains has been investigated and is being prepared for publication.

Significance to Biomedical Research and the Program of the Institute

These studies are intended to contribute to our knowledge of the processes by which both RNA and DNA viruses replicate. The ultimate goal, which is finding means of interrupting the viral replication cycle, will undoubtedly require approaches involving multiple disciplines, including such electron microscopic studies.

The ultimate significance of protozoal viruses is not known. However the possibility that protozoa which are pathogenic for man may represent vectors for certain human diseases which are suspected to be of viral origin must be considered. In view of the failure of attempts to cure bacterial diseases with bacteriophage, it does not seem likely that disease caused by pathogenic protozoa will be amenable to treatment by protozoal viruses.

Proposed Course of the Project

1. Continued effort is being expended to elucidate virus replication by means of electron microscopy coupled with various collaborative studies employing other methods. The

major effort will be on slow viruses such as sheep pneumonitis, Visna and scrapie.

2. Continued intensive study of all phases of the new ameba viruses is planned in collaboration with LPD. Extensive efforts are being made to develop simple and efficient assay systems. We plan to expand the search for protozoal agents to include other pathogens of man, particularly among the trichomonads, and malarial parasites.

Publications:

Takemoto, K. K., Mattern, C. F. T., and Murakami, W. T.: The papova virus group. In Maramarosch, K., and Kinstak, E. (Eds.): Comparative Virology. New York, Academic Press, 1971, in press.

Takemoto, K. K., Mattern, C. F. T., Stone, L. B., Coe, J. E., and Lavelle, G.: Antigenic and morphological similarities of progressive pneumonia virus, a recently isolated "slow virus" of sheep, to Visna and Maedi viruses. J. Virol. 7: 301-308, 1971.

Serial No. NIAID-71-G1
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biological Ultrastructure

Previous Serial Number: SAME

Principal Investigator: Dr. C. F. T. Mattern

Other Investigator: Mr. Wendell A. Daniel

Cooperating Units: Dr. Bronislaw M. Honigberg, Dept. Zoology, University of Massachusetts
Dr. Robert I. Henkin, Clinical Endocrinology Branch, NHLI
Dr. H. G. duBuy, LVD, NIAID, 71-I

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1 3/12		1	2 3/12
Professional	1		1	2
Other	3/12			3/12

Project Description:

Objectives

To study the ultrastructure of protozoa, metazoa, and vertebrates with particular emphasis on the correlation of ultrastructure and function. Organisms and organs under study are selected both to study phylogenetic kinships and certain details of embryogenesis.

Methods Employed

High resolution electron microscopic observations of sectioned cells and organisms is emphasized. In addition organs, cells and isolated cellular organelles are investigated for ultrastructural detail by additional techniques including negative and positive stain, light microscopy with conventional staining, fluorescent antibody staining and dye binding.

Major Findings

1. Continued collaborative studies on the ultrastructure of trichomonads have now included the most primitive of this major order, Monocercomonas. A detailed study of its locomotor organelles (the mastigont complex) has provided further evolutionary clues as to the kinships of these complex unicellular animals.

2. Other studies on the mastigont complex, thru analysis of cinemicrographic films, have revealed that the costa (a major striated rootlet) of Trichomitopsis termopsidis is an undulating locomotory organelle rather than simple the passive, supporting organelle heretofore assumed. Studies are in progress to isolate and identify the biochemical constituents of this organelle.

3. Investigations are continuing on the architecture of different types of human taste bud (circumvallate, fungiform, palatine) in order to evaluate possible cell types involved in the four primary taste qualities. These studies have been recently extended to include the contact chemoreceptors (taste) of the blowfly which receptors apparently contain very few cells about which a great deal is known neuro-physiologically. Attempts are being made to relate the ultrastructural details of the blowfly tast receptors and those of vertebrates.

Continued studies in normal and pathological taste buds have resulted in the recognition of ultrastructural alterations associated with new syndrome (idiopathic hypogusia).

Significance to Biomedical Research and the Program of the Institute

The role of the kinetosome or centriole in a variety of biological systems is well established. Its role in morphogenesis and function of cilia and flagella, formation of spindle and other microtubule systems, and a universal presence in sensory cells render it an organelle of extraordinary interest and of great potential importance. These studies are all designed to shed light on the biological function of the kinetosome.

Proposed Course of the Project

These comparative ultrastructural studies of the anatomy of protozoa, metazoa, vertebrate cells and organs will be continued. They will be correlated with ultrastructural studies of the embryogenesis of certain vertebrate and invertebrate organs.

Publications:

Mattern, C. F. T.: The development of muscle; phylogenetic and embryological considerations. In Laki, K. (Ed.): Contractile Proteins and Muscle Contraction. New York, Dekker, 1971, in press.

Henkin, R. I., Schechter, P. J., Hoye, R., and Mattern, C. F. T.: Idiopathic hypogusia with dysgusia and dysosmia; A new syndrome. J. Amer. Med. Assoc., 1971, in press.

Mattern, C. F. T., and Honigberg, B. M.: Observations in the undulatory motion of the costa of *Trichomitopsis termosidis*. Transactions of the American Microscopical Society, 1971, in press.

Honigberg, B. M., Mattern, C. F. T., and Daniel, W. A.: The fine structure of the mastigont system of *Tritrichomonas foetus* (Riedmuller). J. Protozool, 1971, in press.

Serial No. NIAID-71-H
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Host defenses against viral infection: Interferon and Immunity

Previous Serial Number: SAME

Principal Investigator: Dr. Samuel Baron

Other Investigators: Charles E. Buckler, LVD, NIAID
Dr. Herbert Oie, Guest Worker, LVD, NIAID
Dr. Michael Worthington, Guest Worker, LVD, NIAID

Cooperating Units: Dr. Herman duBuy, LVD, NIAID, 71-I
Dr. Robert Chanock, LID, NIAID, 70-G
Dr. Francoise Cathala, Guest Worker, NINDS
Dr. Robert Herman, LBV, NIAID, 125-Q
Dr. Hilton Levy, LVD, NIAID, 71-F
Dr. Peter J. Gerone, Fort Detrich
Dr. Louis W. Catalano, NINDS
Dr. Aaron E. Freeman, MAI
Dr. Miles Galin, N. Y. Med. College

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3		4 11/12	4 11/12
Professional	2		1 11/12	3 11/12
Other	1			1

Project Description:

Objectives

1. Studies of the mechanisms of production of interferon.

2. Role of interferon during infection.

3. Application of the interferon system to achieve control of viral infections.

Methods Employed

Polyinosinic acid and polycytidylic acid were annealed to form the double-stranded inducer of interferon poly I.poly C. Studies of interferon production were conducted by administration of interferon inducers and then serial sampling of blood and tissues for interferon assays. Interferon was assayed by reduction of yield of interferon sensitive viruses in a one step growth cycle in homologous cell cultures. In the animal protection studies the interferon inducing substances or interferon itself were administered by appropriate routes before or after viral infection and serial samples were collected for assay of virus, interferon and antibody. In the human studies the volunteers received poly I.poly C dropwise intranasally by the dose schedule described under results and were subsequently infected with rhinovirus 13 or influenza A2. Serial samples were collected for virus, interferon, antibody, and toxicology studies. Sensitivity of cells to interferon or to interferon inducers was determined as the smallest amount of the preparation capable of significantly inhibiting growth of interferon sensitive viruses in the appropriate cell culture.

Major Findings

1. Mechanisms of production of interferon. The kinetics of in vivo interferon response to the synthetic double-stranded RNA, poly I.poly C was studied. In mice a single intraperitoneal injection of poly I.poly C induced an interferon response with peak titer and total duration proportional to the amount of inducer injected. Repeated injections within the first 48 hours after the initial induction increased the total duration of production by about 12 hours without increasing the peak titer. The ability to obtain high, sustained levels of interferon was hampered by hyporesponsiveness to restimulation beginning 36 hours after the initial stimulation. The nature of this hyporesponsiveness was further studied and is discussed in the summary of current work presented by Dr. H. duBuy. By suitable adjustment of the dose schedule of poly I.poly C sustained, moderate levels of serum interferon were achieved for a period of 7 or more days.

A study was undertaken to compare interferon levels in rabbit brain, cerebrospinal fluid and serum following injection of poly I.poly C. After intravenous administration the titers of interferon in the brain and cerebrospinal fluid were always a small fraction of those in the serum. Intrathecal administration of poly I.poly C significantly increased interferon in the cerebrospinal fluid but did not significantly affect the interferon titers in the brain. Injection of poly I.poly C intracerebrally did give rise to more sustained levels of interferon in the brain. However, the highest levels of brain interferon were always far below the highest levels which could be achieved in the serum. Local production of interferon in the brain and its meninges was indicated by the finding that after intracerebral administration of poly I.poly C the levels of interferon in the brain and cerebrospinal fluid exceeded those in the serum.

In a related study systemic or intraocular administration of poly I.poly C in the rabbit produced measurable interferon levels in the serum, acqueous fluid of the eye and vitreous fluid of the eye. Intraocular levels of interferon were greatest with intravenous administration of poly I.poly C. The injection of poly I.poly C into either the anterior chamber of the eye or the vitreous cavity produced significant intraocular levels of interferon, indicating that the eye is a source for interferon production. Highest levels of vitreous interferon were produced after intravitreal injection of poly I.poly C.

2. Role of interferon during infection. A possible role of the interferon system during infection of mice with the protozoal parasite Leishmania donovani was examined. Depending upon the conditions of induction and the timing there was enhancement or inhibition of parasite multiplication. The altered parasite levels could not be related to interferon. These findings are discussed in detail in the annual report of Dr. Robert Herman (LPD).

The interferon system was evaluated in fibroblasts from persons predisposed to leukemia or other cancers, in fibroblasts from persons with neoplastic disease, and in human tumor cells. Of 31 normal fibroblast strains from patients with tumors or diseases associated with a high incidence of malignancy, only one cell strain had a poor response to either of the two interferon inducers used, polyinosinic.polycytidylic acid and Chikungunya virus. On the other hand, cell cultures of five human tumors were significantly less sensitive to the antiviral effect of these interferon inducers and human interferon. In addition they produced less interferon in response to Chikungunya virus than any of the nontumor tissues studies.

A number of normal rat cell cultures as well as cultures transformed spontaneously, by chemicals, and/or by oncogenic viruses were also tested for responsiveness to the interferon inducer polyinosinic.polycytidylic acid, or to exogenous interferon. Responsiveness, or lack thereof, had no correlation with subculture passage number, infection with RNA leukemia virus, morphological transformation by oncogenic RNA or DNA viruses, chemical treatment, or the ability of these cells to produce tumors in isologous host animals. The data indicate that lack of response to interferon or to the inducer is niether a necessary prerequisite nor an absolute result of cellular transformation of rat cells.

3. Application of the interferon system to control viral infections. Poly I.poly C, an inducer of interferon, was administered dropwise, intranasally to volunteers for a 7 day period beginning 1 day prior to inoculation with rhinovirus 13 or influenza A2/Hong Kong/68 virus. In a total of three separate trials, a small but definite reduction in symptoms of upper respiratory illness was associated with poly I.poly C treatment. However, there was a variable affect on the pattern of virus infection. In only one of the two rhinovirus studies was reduction of virus shedding associated with poly I.poly C treatment. In addition, treatment did not decrease the shedding of influenza A2 (Hong Kong) virus. Serum and nasal antibody response to the challenge viruses was not altered by the treatment. The minimal amounts of nasal interferon stimulated by the intranasal poly I.poly C treatment may have been responsible for the less than optimal results obtained. Toxicity was not detected. These findings will be discussed additionally in the annual report of Dr. Robert Chanock, LID, NIAID.

Aerosols of poly I.poly C were useful in protecting mice infected with aerosols of influenza (A2/Taiwan/64) and parainfluenza type 1 (Sendai) viruses. Administration of poly I.poly C as an aerosol offers an advantage particularly in therapy by eliminating the risk of pulmonary dissemination of viral infections due to intranasally instilled fluids. Pretreatment of mice with aerosols of poly I.poly C reduced the infection rate with influenza virus but did not inhibit virus multiplication in the lungs of those animals where infection became established. Sendai virus infection rates were undiminished in mice pre or post treated with poly I.poly C but virus titers in the lungs were significantly suppressed as compared with those of untreated animals. The maximum poly I.poly C doses (40 ug) administered by aerosol produced no evidence of toxicity in the mice. This study was done in collaboration with Dr. Peter J. Gerone, Fort Detrich.

Using the information developed in the study of kinetics of production of interferon in the mouse following poly I.poly C administration a number of different dose schedules of poly I.poly C were compared in Semliki Forest virus infected mice. Daily treatment with 100 ug intraperitoneally was more effective than any other of the dose schedules tested. Using this regimen, it was possible to significantly decrease the mortality of Semliki Forest virus infected mice when treatment was begun as late as five days after virus inoculation, at a time when high titers of virus were present in most brains. In experiments where the interferon stimulator gave rise to more interferon than had been applied passively in previous studies, the inducer exerted a much greater protective effect.

The interferon sensitivities of intracerebrally administered herpes simplex virus and encephalomyocarditis virus were determined in mice using poly I.poly C as the interferon inducer. The protective effect of poly I.poly C against intracerebral infection of mice with HSV and EMCV was equal only when the challenge dose of virus was sufficiently low. The protective effect against HSV was lost with increasing virus doses wherease the protective effect against EMCV was decreased only slightly with increasing virus dose.

A study of the comparative antiviral action of interferon, interferon inducers and IDU against herpes simplex virus infection of the rabbit eye was done. Rabbit eyes were treated topically with poly I.poly C, pyran copolymer rabbit serum interferon, or idioxuridine beginning the day prior to inoculation with herpes simplex virus. Poly I.poly C and idioxuridine provided effected prophylaxis against keratoconjunctivitis. Pyran copolymer and interferon were ineffective in the doses employed. In a related study topical poly I.poly C was used prophylactically and therapeutically in experimental vaccinia keratitis of rabbits. It was found that this interferon inducer protected against vaccinia infection when administered from three days up to 8 hours before infection. Its therapeutic efficacy diminished with time and was absent 24 hours after infection. These studies were done collaboratively with Dr. M. Galin at New York Medical College.

Significance to Biomedical Research and the Program of the Institute

The studies of interferon production in vitro and in vivo have helped elucidate the controlling mechanisms for the body's interferon system and for the responsiveness of different viruses to this antiviral system. The information gained was utilized to maximize the induction of interferon in experimental animals. Using the improved responsiveness of the interferon system an evaluation was made in animals of the degree of protection which could be achieved prophylactically and therapeutically against a variety of viral infections. Aerosol administration to mice of interferon inducers demonstrated that inducers could be safely administered to the lung in aerosol fashion without increasing the severity of preexisting influenza pneumonia. In fact such administration was shown to be therapeutic in that administration of poly I.poly C by aerosol one day after parainfluenza virus infection of the mouse lung resulted in a significant protective effect. An optimum dose schedule of poly I.poly C administered systemically could protect against an arbovirus encephalitis as late as five days after infection when the virus already had achieved very high titers in the brain. Comparative prophylaxis and therapy studies of rabbit eye infections showed that strong interferon inducers were more effective than were the presently available amounts of rabbit interferon. Vaccinia keratitis also responded to the interferon inducer but the protective effect (as with Herpes simplex) diminished by one day after the onset of infection. The first trials of poly I.poly C intranasally in man against a rhinovirus and influenza virus demonstrated a small but definite protective effect which was correlated with induction of small amounts of interferon. These results are encouraging and indicate that more effective schedules of interferon inducers or more effective inducers need to be developed to increase the interferon response in man and thereby increase the protective effect to the levels which can now be achieved in other species.

The finding that human sarcoma cells in tissue culture have an abnormally low interferon function supports the possibility that an abnormality of the interferon system may be related to development of certain human sarcomas. However, a parallel study in transformed rat embryo cells did not demonstrate this defect. The latter finding reinforces the conclusion that if diminished effectiveness of the interferon system is related to tumorigenesis, then it is not a general correlate.

Proposed Course of the Project

Studies of the mechanisms of production of interferon will continue to help delineate the controlling factors which operated in vitro and in vivo as well as to establish optimal dose schedules for induction of interferon in protection studies in animals and man. The existing hypotheses on the relative roles of interferon, antibody and cellular immunity during virus infection will be tested with the use of immunosuppressive substances, attempted induction of immune tolerance, passive administration of interferon, induction of interferon, passive transfer of antibody and passive transfer of sensitized lymphocytes. Studies to better control viral infections in animal systems will continue with improved dose schedules, evaluation of new inducers of interferon and the development of methods to help overcome hyporesponsiveness to repeated induction of interferon. The search for a more suitable line of human cells to serve for assay of human interferon will continue since no fully acceptable cell line is presently available. Studies will be continued in collaboration with the international groups working to establish reference standards for interferon assays.

Honors and Awards:

(1) Associate Editor, American Journal of Epidemiology

(2) Associate Editor, Infection and Immunity

(3) Member of the program committee for the annual meeting of the American Association of Immunologists.

(4) Member of the educational committee of the American Association of Immunologists.

(5) Invited lectures at Universities, Research Institutes, and Symposia.

Publications:

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Serial No. NIAID-71-I
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The interaction between latent and slow viruses, especially the Lactic Dehydrogenase Virus (LDV) and the subcellular elements of the host cell (mouse macrophages for LDV). Use of the LDV-modified macrophages model for the study of role of macrophages in the defense mechanisms of the host.

Previous Serial Number: SAME

Principal Investigator: Dr. Herman G. duBuy

Other Investigators: None

Cooperating Units: Dr. C. F. T. Mattern, LVD, NIAID, 71-G
Dr. Samuel Baron, LVD, NIAID, 71-H
Dr. Robert Herman, LPD, NIAID, 125-Q
Mr. M. Lunde, LPD, NIAID, 125-P

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2			2
Professional	1			1
Other	1			1

Project Description:

Objectives

Study of the factors, both in vivo and in vitro, which determine whether a virus kills the host, becomes chronic, or is eliminated, and of factors which modify the outcome of the virus infection: i.e., interferon, humoral antibody and cellular defense factors, especially those of macrophages, the target cells of LDV.

Methods Employed

The Murine Lactic Dehydrogenase virus is used as a model because:

(a) It is an RNA-membrane virus which multiplies in the cytoplasma of mouse macrophages, the only target cell for LDV.

(b) It leads to a chronic viremia, which is modifyable by interferon and its inducers, by antibody, and by cellular defense factors.

(c) It can be titered by measuring plasma enzyme elevations, caused by persistent decreased clearance of 4-6 enzymes from the blood, which is characteristic for a change in macrophage function.

Major Findings

(1) Single and repeated doses of the interferon inducer poly I.poly C, during both the acute and chronic stage of infection, led to a decrease in viremia. Various regimens to eliminate the viremia are under study.

(2) Prolonged treatment of mice with the immunosuppressive drug cyclophosphamide led to a decrease in LDV-antibody titer, with a concomitant increase in viremia during the chronic stage.

(3) Mice infected with Besnoitia jellisoni were not resistant to challenge with LDV; neither were their macrophages, cultivated in vitro.

(4) Mice with other chronic protozoal infections, Leishmania donovani and Plasmodium berghei, were found to be resistant to challenge with LDV, as were their macrophages cultivated in vitro.

(5) Certain intracellular protozoal infections (Besnoitia jellisoni, Leishmania donovani and Plasmodium berghi) led to an elevation of plasma-LDH enzyme levels, and to a marked potentiation of the LDH enzyme levels, caused by LDV infection.

The findings indicate how three sets of defense mechanisms, which determine the degree of LDV viremia: interferon formation, humoral defense and cellular defense, can be modified by application of interferon or its inducers, by immune suppressive substances and by heterologous infection, which changes cellular defenses, especially of macrophages.

Significance to Biomedical Research and the Program of the Institute

The study of the chronic viremia caused by LDV contributes to the understanding of other chronic viremias and the possibility of their cure. This holds especially for those where a lifetime viremia is found, and where infectious antigen antibody complexes have been demonstrated, as in LDV. Other examples are: the chronic viremias caused by lymphocytic choriomeningitis, serum hepatitis, murine and chicken leukemia, Aleutian disease of mink, scrapie and Visna. The use of the LDV macrophage model further contributes to an understanding of the factors underlying defense mechanisms against other chronic diseases.

Proposed Course of the Project

Continuation of the studies to decrease or eliminate chronic LDV viremia, by using interferon inducers with differing properties, and by varying the regimens of induction. Continuation of the studies with the macrophage LDV model to determine changes in macrophages function, either in vivo or in vitro, brought about by other intracellular parasites, especially in relation to their effect on the cytoplasmic locus of LDV replication.

Honors and Awards:

(1) Member of the "Neurological Disorders Program Project Review Committee", NINDS, Johns Hopkins University Medical School, Baltimore, Maryland, December 27-31, 1970.

(2) Guest lecturer at the Biology Department of the Catholic University, Washington, D. C., February 1, 1971.

Publications:

duBuy, H. G., Johnson, M. L., Buckler, C. E., and Baron, S.: Relationship between dose size and dose interval of polyinosinic.polycytidylic acid and interferon hyporesponsiveness in mice. Proc. Soc. Exp. Biol. Med. 135: 340-344, 1970.

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Serial No. NIAID-71-J
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tumor Viruses and Cells

Previous Serial Number: SAME

Principal Investigator: Dr. Kenneth K. Takemoto

Other Investigators:
Dr. Lawrence B. Stone, LVD, NIAID
Dr. C. F. T. Mattern, LVD, NIAID
Dr. John E. Coe, RML, NIAID
Dr. M. A. Martin, LBV, NIAID
Dr. Edward Scolnick, NCI

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3			3
Professional	2			2
Other	1			1

Project Description:

Objectives

To study tumor viruses and cells from a broad standpoint in order to elucidate mechanisms involved in viral carcinogenesis. These investigations are concerned with lytic and abortive infection in permissive or non-permissive hosts, viral genetics, and properties of transformed cells.

Methods Employed

Standard cell culture and virologic methods are used.

Major Findings

In the past, this laboratory has employed the small DNA-containing oncogenic papovaviruses as models for the study of viral carcinogenesis. While studies utilizing these viruses are still continuing, recent developments concerning the RNA-containing tumor viruses and their possible relationship to certain "slow viruses" has resulted in a change in the direction of research in this laboratory. The major findings in this new area of research can be summarized as follows:

1. RNA-dependent DNA polymerase in Visna virions. Visna, a virus isolated a number of years ago from sheep with a slow, demyelineating disease was found to contain a "reverse transcriptase" within the virion, much like the RNA tumor viruses. This enzyme makes a DNA copy of the RNA genome and is believed to play an important role in the process of transformation by RNA tumor viruses. Like the RNA tumor viruses, visna virus also contains another enzyme, an endonuclease, within the virus particle.

2. Transformation by Visna and progressive pneumonia virus (PPV). Since the RNA-dependent DNA polymerase is thought to be important in viral oncogenesis, it was reasoned that any virus possessing such an enzyme may have the capacity to transform cells under suitable conditions. Visna virus was therefore tested for ability to transform cells in culture. Mouse cells were found to be susceptible to neoplastic transformation by visna and progressive pneumonia viruses. The transformed cells possessed properties generally associated with viral transformation, i.e., morphological alterations, loss of contact inhibition of growth and movement, ability to cause tumors when inoculated in syngeneic animals, and the presence of rescueable genome. Sheep testis cells which are permissive cells for growth of these viruses can be chronically infected and after a prolonged period, these cells can also become morphologically transformed and be endowed with the capacity for indefinite growth.

3. Relationship of progressive pneumonia virus to visna and maedi. Progressive pneumonia virus was isolated by workers at the Rocky Mountain Laboratory a few years ago from sheep affected with a slow pulmonary disease. Although the virus appeared to have some properties in common with visna and maedi, its exact identity was not established. Serologic studies in this laboratory using the fluorescent antibody procedure definitively proved that the progressive pneumonia virus was related to visna and maedi viruses. Electron microscopic studies showed that the virus matured by a budding process from the plasma membrane, much like the C-type RNA viruses. Extracellular forms were found to be about 80 to 100 nm in diameter with a dense internal nucleoid of about 30 nm.

The nucleoid in free particles was frequently eccentrically displaced and resembled the virions of mammary tumor virus. Cytoplasmic forms somewhat like the A particles of mammary tumor virus were also seen in infected cells.

4. Biophysical characterization of progressive pneumonia virus. Since both visna and progressive pneumonia viruses bore striking resemblances with the RNA tumor viruses, further studies were done to characterize the virions of these viruses. PPV was purified by centrifugation in sucrose density gradients. The virus had a buoyant density of 1.16 to 1.18. The RNA extracted from purified virus consisted of large 60 to 70S molecules together with lesser amounts of 36S and 4S molecules. These results are consistent with what has been found in RNA tumor viruses and strengthens the current ideas about the possible taxonomic grouping of these viruses with the RNA tumor viruses.

Significance to Biomedical Research and the Program of the Institute

The findings on the relationship of the slow viruses to the oncogenic RNA viruses may have important implications in explaining mechanisms of the disease processes in slow viral infections and carcinogenesis. These studies have shown that the genome of visna or PPV can become "integrated" with host cells under experimental conditions; such an integration mechanism may explain the slow course of infection by these viruses. It is possible that certain slow viral infections of man (Kuru, multiple sclerosis, etc.) may be approached with the newer techniques developed in the studies with visna or the RNA tumor viruses.

Proposed Course of the Project

1. Transformation studies by visna and PPV will be continued with an attempt to determine the host range of these viruses. Other studies will deal with the status of the genome in transformed cells, possible new virus-specific antigens in the cells (including transplantation antigens), techniques for "rescue" of viral genome from non-releasing cells, etc.

2. The virions of these viruses will be further analyzed. The proteins of the virus are currently being studied together with the new enzymes. In collaboration with others, it is hoped that the DNA product made by the polymerase may be compared with those of the RNA tumor viruses for possible relatedness.

3. The National Cancer Institute is currently conducting extensive screening tests for inhibitors of the RNA-dependent DNA polymerase. Some of the compounds already shown to be

potent inhibitors of the enzyme (for example, N-demethyl rifampicin) will be tested for inhibition of visna virus in cell culture. Since a convenient and precise assay is available for visna, this virus may be better suited than the RNA tumor viruses for screening chemotherapeutic compounds.

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LABORATORY OF MICROBIOLOGY

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PHS-NIH
Summary Statement
Laboratory of Microbiology, NIAID
July 1, 1970 through June 30, 1971

Introduction

At the start of the fiscal year, we established an Immunology Section staffed by reassignment of Mr. Julius Goode and Dr. Sanford H. Stone, Section Head. On July 1, Dr. Alan Glick (Staff Associate) came on duty. In October, Miss Virginia Burlingame (Secretary to the Chief, LM) retired after over 30 years of federal service and was replaced by Mrs. Linda G. Barbine. Miss Judith Moir (Clerk-steno, Mycoplasma Section) came on duty in November. In April, Dr. Susan Birnbaum completed 2 years as an NIH Post-Doctoral Fellow. In June, Mr. James Turner (Biol. Lab. Tech.) and Dr. George Lones (Scientific Director, USPHS) retired with 29 and 20 years of service, respectively; and Dr. Richard Getnick completed duty as a Staff Associate. Dr. Roger M. Cole presented the Annual Sigma Xi Lecture at the University of South Dakota in November. Dr. Joseph G. Tully served as a member of the International Subcommittee on Taxonomy of the Mycoplasmatales; and of the WHO/FAE Board of Animal Mycoplasma Characterization in Paris in March.

Although scattered among the Sections according to scientific discipline or nature of the microorganisms studied, the research interests of this basic laboratory reflect several areas of broad microbial importance. These include genetics (both bacterial and fungal); metabolism and the relation of structure and function; the nature and reactivities of microbial antigens and haptens; the nature, identification, and taxonomy of non-walled microorganisms; fungal ecology; some aspects of pathogenesis; and the nature and mechanisms of immune responses, particularly hypersensitivity. Selected brief summaries of research findings in some of these areas are presented below.

Bacterial Structure and Function Section

Importance of Carbon Dioxide in Bacterial Growth

Precise control of gases in a special apparatus showed that CO_2 concentration, in its balance with bicarbonate ion, is critical in growth of both autotrophic (Hydrogenomonas eutropha) and heterotrophic (Escherichia coli: Group H Streptococcus) bacteria. The proper concentration completely eliminated the usual lag period. However, the primary requirement of H. eutropha was bicarbonate ion, whereas that of the streptococcus was CO_2. The consistency of the CO_2-bicarbonate effect in different sorts of bacteria, if widely confirmed, has important implications in production of microorganisms and in basic questions of bacterial growth. (Repaske; Guest workers A. Repaske and DeCicco)

Protein Differences of Bacterial Membranes and Mesosomes

Cytoplasmic membranes and mesosomal vesicles of both streptococci and staphylococci were isolated and separated by chemical and physical methods previously described. By gel electrophoresis, more and differently-patterned protein bands were obtained from membranes than from mesosomes of streptococcu Also, fewer protein bands, which excluded those of higher molecular weight, were obtained from electrophoresed extracts of intact cells as compared with lysed cells--suggesting an extraction restriction by cell wall. Staphylococcal membranes and mesosomes showed equal numbers of protein bands, but patterns and densities were different. In either organism, membranes versus mesosomes show no qualitative differences to date in lipids, carbohydrates, or nucleic acids; and quantitation is in progress. The protein differences may be of importance in function and structure, and detailed examinations of protein natures and enzymatic activities are being made in order to define postulated differences in the roles of these bacterial organelles. (Schade, Theodore, Popkin, Cole, Panos [Einstein Medical Center, Philadelphia])

Membrane Glycolipids in Bacterial Serology

Previously, the antigenic determinant (hapten) in important serologic reactions of *Mycoplasma pneumoniae* was shown to be glycolipid in nature. Improved procedures have now allowed reaggregation of purified *M. pneumoniae* glycolipid with membrane protein of *Acholeplasma laidlawii* to form ultrastructurally typical, but hybrid, membranes of improved immunogenicity and *M. pneumoniae* specificity. These offer a new potential for prevention of "atypical pneumonia" in humans if ongoing protection tests in animals are successful. In addition, membrane glycolipids common to both parental bacteria and L forms of some streptococci were purified and shown to determine, and to cross-react in, several different serologic tests. Intensive study of membrane glycolipids of bacteria, L forms, and mycoplasma is in progress in order to determine their natures, interrelationships, and importance in serologic reactions. (Prescott, King, Popkin, Birnbaum [P. D. Fellow], Cole, Caldes, Whitt--all LM; Chanock, James, Razin [Visiting Sci.]--all LID)

Group G Streptophage, Lysing Other Groups, is also a Transducing Phage.

Bacteriophages of streptococci have been considered specific for the host serogroups. Last year, however, we noted that the first known isolate of a Group G (temperate) phage (GT 234) lysed and lysogenized several representatives of streptococcal Groups A, C, and G. This isolate has now been clearly shown to be a single phage by controlled studies of cross-absorption, plaque formation, cross-lysogenization, cross-neutralization, and progeny titers, when passed in susceptible strains of the several host serogroups. Further investigations showed that a morphologically and serologically related Group A virulent phage (A25), as well as a Group A temperate phage (AT 298), can infect some strains of Group C but not of Group G. Both A25 and AT298 are transducing phages for Group A, and now GT234 has also been shown to transduce streptomycin-resistance within Group A. Intensive current investigation is directed to production and detection of inter-group transduction, in the hope of eventually explaining variations in streptococcal antigens, virulence, and other attributes. (Colon, Leonard, Cole)

Progress Made in Defining Genetic Competence in Streptococci

The nature of the ability of some bacterial cells to take up and integrate genetic information of externally added DNA (genetic competence) is incompletely known. Our recent studies of the Group H streptococcal system now permits better definition of the stages involved. The first step is production by the bacteria of a soluble extracellular competence factor (CF)--the reattachment of which to the cells initiates all other processes of competence. We have now shown that, in a defined medium, CF can be produced independently of subsequent steps by a strain that never becomes competent in the same medium but that can be made competent by the sole addition of glutamate. CF bound to cells can be removed at 60°C and pH 2.0, is dialyzable, has a molecular weight of about 5,000, and is inactivated by trypsin. It is homogeneous by gel and paper electrophoresis, and isoelectric focusing shows 6 bands, one of which contains the bulk of the biological activity. Analysis of this band is in progress, and will lead to clarification of the succeeding step. In this step, CF binds to relatively uncharacterized receptors of the bacteria and somehow initiates further steps known to be chloramphenicol-sensitive and to therefore require protein synthesis. This past year, we showed, by acrylamide-gel electrophoresis, the appearance of a new protein at this stage. At the same time, our studies demonstrated lysis, under certain conditions, that was restricted to the cell site of bacterial division. Both phenomena were seen in competent cells, but not in non-competent; and both were prevented by chloramphenicol as was subsequent competence (i.e., DNA uptake). Supernates from lysed competent cultures could lyse non-competent cells. These findings raise again the question of role of cell-wall autolytic enzymes, which has been invoked in other bacterial competence systems, and which has been postulated necessary to make holes in the cell wall for entry of the large DNA molecules. The relation of new protein to autolysin, and the nature of autolysin, are under continuing study. (Leonard, Ranhand, Cole)

Problems of L Form Stability Examined

A stable (non-revertable) bacterial L form (F24L) has been examined in several attributes, in attempts to relate stability to a previously-demonstrated genomal defect. In comparison with the parent organism (Streptococcus faecium F24), the L form lacks both the peptidoglycan (murein) "backbone" and serologically-characterizable polysaccharide polymers of the cell wall. Neither form can be genetically mapped by sequential mutation because of their failure to synchronize division in the requisite complex medium after exposure to inhibitors of DNA replication. The physical association of DNA and membrane, presumed essential to orderly DNA replication and segregation, could not be shown by isolation of DNA-membrane complexes from the L form. Other peculiarities include the stability of F24L to centrifugation (reported to kill some other L forms); and the failure of an "osmotic stabilizer" (ammonium chloride) which was successful in one medium, to support F24L integrity in another. These diverse findings emphasize the difficulties in assessing a basis for L form stability. (King)

Intraphagocytic Degradation of Group A Streptococcal Cell Walls Studied

A number of reports have indicated the persistence of Group A streptococcal cell walls sequestered in macrophages within tissues of injected animals.

Results were similar whether intact cells or isolated walls were injected, and the obvious question arose as to whether any cell wall degradation occurred. To try to answer this, human monocytes (i.e., macrophages) from peripheral blood were obtained by a density-centrifugation method, allowed to ingest streptococci in an in vitro system, and examined at intervals chemically and by electron microscopy. Rhamnose, as the principal and probably most available component of the streptococcal wall, was selected for chemical detection on the assumption that its presence in particle-free supernates of disrupted phagocytes after streptococcal ingestion would be evidence of wall degradation. After many modifications to eliminate interfering substances, a method involving acid hydrolysis and enzymatic conversion was devised to measure as little as one microgram of rhamnose. For 5 micrograms or more, paper chromatography was satisfactory. The revised techniques are now being applied in the experimental system. Meanwhile, electron microscopy showed bacteriolysis but no detectable breaks nor thickness changes in cell walls of phagocytosed streptococci in monocytes. More extensive changes were seen within polymorphonuclear leukocytes, but complete wall degradation was not seen in either system during the in vitro phagocyte survival time (6-8 hours). A comparison of phagocytosis of isolated cell walls and mucopeptide "shells," with the aid of the newer analytic methods, is expected to supply answers important in the pathogenesis of post-streptococcal disease. (Getnick, Glick, Cole)

Medical Mycology Section

Genetics of Blastomyces dermatitidis (Ajellomyces dermatitidis) Clarified

This is the only fungus, among those causing the more common systemic mycoses, that can be subjected to genetic analysis by conventional mating procedures. Studies have shown that there is a direct relationship between colonial pigmentation and competence for mating with the opposite type. Strains possessing the buff pigment mate readily with their compatible tester strain, whereas, strains that are non-pigmented or white do not. From studies of single ascospore isolates of F_1 and F_2 progeny and an analysis of their competence for back crossing, it is concluded that mating in this fungus is controlled by a bipolar incompatibility system. Future studies will be directed towards the genetic control of dimorphism. (Kwon-Chung)

Ecology of Histoplasma Remains a Riddle

The occurrence of limited foci of Histoplasma capsulatum in soils of certain ecological states (e.g., chicken yards, bat roosts) is accepted, but the conditions restricting or favoring the fungus are poorly defined. Our current study incriminates the nature of the soil microflora. High total bacterial counts, and low counts of other fungus-like organisms (streptomycetes) coincide with the presence of H. capsulatum; whereas negative soils show the reverse. A condition (addition of bat guano to soil), which was shown in the laboratory to favor the growth of H. capsulatum, also increased bacteria but not streptomycetes. Nevertheless, similar guano applications to a natural site did not increase recovery of H. capsulatum in an 18 month period. We have also shown persistence of H. capsulatum in a fixed, limited natural focus without extension for 12 years: in another, following a prior drastic decrease of unknown cause, the level of recovery has been stable for 3 years. The many unexplained variations necessitate ongoing further attempts to define factors in influencing the natural occurrence and persistence of this fungal pathogen of man. (Hasenclever, Kwon-Chung)

Pathogenesis and Kidney Invasion by *Aspergillus bisporus* Shown

This newly described species is unique among known fungal pathogens because of its initial predilection for the kidneys of mice. Histologic examination of kidney tissue taken 4,6, and 22 hours after infection showed spores in both glomeruli and outer cortex. Four to 9 days after infection, spores were observed in the inner cortical area but after 2-3 weeks no spores or fungal growth could be detected in the cortex or Bowman's capsule. Extensive hyphal growth and necrosis, however, was apparent at this time in the tips of the papillae and in the pelvic area. The site of proliferation is distinct from that of other fungi which may invade the kidney secondarily. Currently, studies are in progress to determine if a strain of mice with congential primary polydipsia are less susceptible to infection, possibly due to a lowered osmolarity of their urine, than mice with normal kidney function. (Kwon-Chung)

Mycoplasma Section

New Sterol-Nonrequiring Mycoplasmas

Earlier studies revealed two mycoplasmas capable of growing in a sterol-free environment. Each was shown to be serologically distinct from the single *Mycoplasma* serotype heretofore known to lack a sterol requirement. These observations led to recent taxonomic proposals to reclassify the sterol-nonrequiring mycoplasmas as species of a new genus, *Acholeplasma*. We have characterized and described one of the new serotypes as *Acholeplasma axanthum*. Studies now in progress indicate that the *Acholeplasma* group may represent a much larger number of distinct species than was originally thought. Two new strains (one of swine origin and the other from a tissue culture line) are under current study, and appear to possess properties similar to those of other acholeplasmas. Both cultures lack a sterol requirement, do not possess pigmented carotenoids, and are serologically distinct from the three other acholeplasmas. The mechanisms whereby mycoplasmas are able to synthesize cholesterol in the cell membrane, especially in the absence of free cholesterol and without the formation of carotenoids, is of considerable importance in understanding lipid biosynthesis. (Tully)

Biological and Serological Characterization of New Animal Mycoplasmas

The characterization of various monkey mycoplasmas reported last year has confirmed the relationship of a large number of these isolates to an unclassified *Mycoplasma* (strain Navel). Further analysis of various substrains of the Navel culture and several monkey isolates by acrylamide gel electrophoresis, sterol requirements, biochemical properties, and reciprocal serological tests indicate general identity. A description of the properties of these strains and their proposed designation as *M. primatum* is in preparation.

Additional studies have been carried out on a group of eight mycoplasmas recovered from a variety of animal hosts, including sheep, goats, bovines, swine, and a single human isolate. All of these mycoplasmas show a partial relationship to some pathogenic goat mycoplasmas. A variety of serological and biological tests have been performed to establish their interrelationships. (Tully; Barile, LBP-DBS)

Plate Immunofluorescence and Gel Electrophoresis as Rapid Diagnostic Aids in Mycoplasma Typing

The fluorescent antibody test using agar-grown mycoplasmas and conjugated type-specific antiserum has received extensive use this year in typing tissue culture isolates. We have examined a large number of mycoplasmas recovered from primary mouse tissue cultures, as well as other types of primary lines. The plate fluorescent test has shortened the time required for typing and conserved significant quantities of materials normally required. Typing of mycoplasma contaminants can provide important information on the possible origin of the contamination and indicate whether the flora of primary tissues are involved or exogenous tissue culture materials are contaminated. The gel electrophoresis of mycoplasmal proteins continues to provide important confirmation of serological results. Gel patterns have been obtained on most mycoplasmas in our collection and comparisons are now being made on groups of distinct species with similar GC (guanine-cytosine) ratios. Those species with similar patterns and base compositions values may yield important findings in nucleic acid hybridization tests. (Tully)

Immunology Section

Studies on the Genetics and Mechanisms of Specific Immune Responses of Guinea Pigs

Previous studies disclosed that the response (Strain 2) or non-response (Strain 13) of inbred guinea pigs to dinitrophenol-polylysine was a quantitative rather than an all-or-none phenomenon; so-called "non-responders" respond when appropriate conditions and adjuvants are selected for immunization. A simplistic view (one gene determined response or non-response) was further ruled out when it could be shown that the high and low response occurred also to various protein antigens indicating a general deficit rather than a "Polylysine gene" deficiency in the Strain 13 guinea pigs. (Stone)

Remissions and Relapses in Chronic Autoimmune Encephalomyelitis

Post-vaccinal and post-infectious encephalomyelitis are acute diseases, in contrast to the multiple sclerosis pattern which is chronic and relapsing. Experimental autoimmune encephalomyelitis (EAE), a fair model for the former syndromes, may also have forms which would be better models for MS. Previous work on a chronic EAE which was age- and strain-dependent was extended in long-term attempts to see if surviving animals might show the remissions (and relapses) of a disease even more reminiscent of human demyelinating diseases. Of three immunosuppressive approaches (cyclophosphamide treatment, surgical removal of antigen-adjuvant depots, and immune deviation), the last two might encourage a remitting, relapsing pattern. However, only a small percentage of guinea pigs showed an intermittent type of chronic disease. (Stone)

Cutaneous Basophil Hypersensitivity to Microbial Antigens

Previous work in this section on the adoptive transfer of cutaneous basophil hypersensitivity (or Jones-Mote reaction) showed that this cellular manifestation of an immune response could be separated and distinguished from classic delayed hypersensitivity by appropriate selection of the time of transfer of cells. Injection of living lymph node cells into normal recipients only a few days after immunization of donors with M. tuberculosis in water-in-oil emulsion transfers the Jones-Mote hypersensitivity, whereas a wait of about two weeks is optimal for the classic tuberculin response. It has also been shown that incomplete Freunds adjuvant is a sufficient vehicle for inducing cutaneous basophil hypersensitivity although the whole mycobacterium is necessary for inducing delayed hypersensitivity. When PPD is injected in incomplete Freunds adjuvant several days prior to injection of complete Freunds adjuvant containing killed M. tuberculosis, there is evidence of an immune deviation in favor of the more ephemeral Jones-Mote reactivity. (Stone)

Serial No. NIAID 61
1. Microbiology
2. Bacterial Structure
and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Electron Transport System in an Autotrophic Hydrogenomonad and the Effect of CO_2 on Growth.

Previous Serial Number: Same

Principal Investigator: Dr. Roy Repaske

Other Investigators: Guest Workers
- Dr. Benedict De Cicco
- Mrs. Anne C. Repaske

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	27/12	-	10/12	37/12
Professional	12/12	-	10/12	22/12
Other	15/12	-	-	15/12

Project Description:

Objectives:

1. Investigate the effect of CO_2 (bicarbonate) on the lag period of autotrophically grown *Hydrogenomonas eutropha*.

2. Determine the effect of CO_2 (bicarbonate) on the lag period of heterotrophic bacteria.

3. Complete purification and characterization of hydrogenase from *H. eutropha*.

4. Begin isolation and purification of the second sequential enzyme in the electron transport system: NADH-cytochrome c reductase.

Methods Employed:

The method to determine a CO_2 effect on growth required complete control of the CO_2 available to cells. A more difficult aspect was removal of CO_2 metabolically produced within cells. A 1 liter fermentor fabricated for these experiments was equipped with three separate gas lines through which a large excess of high purity cylinder gases were bubbled through the culture. Individual flow meters controlled the flow rate of the gases and thereby established the percentage of each gas in the mixture. The excess gases being swept through the medium provided assurance that the gas composition was not altered by relatively small gas uptake rates of cells. The dissolved gases in the medium also provided an equilibrium differential which promoted removal of any CO_2 which might be generated within the cell. Gas composition, medium composition, pH control and temperature control are variables which influenced results.

Major Findings:

Subcultures of microorganisms invariably have a lag period before logarithmic growth begins. This is such a normal state of events that the classical growth curve in all microbiology texts shows a lag phase which is euphemistically called "the phase of adjustment". Modern microbiological literature offers an explanation for one type of adjustment. When the inoculum is transferred to a medium different from the one in which it was grown, the organisms may require time to synthesize adaptive enzymes required for the metabolism of the new substrate. No definitive explanation has been given to account for the lag period when the inoculum is transferred into the same type of medium as the one in which it was grown. Among explanations favored from time to time have been : (1) time required to dilute out intracellular metabolic toxins; (2) time required to produce essential intermediary metabolites; and (3) time to rejuvenate 'aged' cells.

1) When we were growing large cultures of Hydrogenomonas eutropha we experienced long lag periods. In attempts to find conditions to minimize the lag period, we discovered CO_2 to be critical. With the proper CO_2 concentration, the lag period was completely eliminated. Additional experiments showed that the CO_2 required depended upon the physiological age of the inoculum. Inocula in the log phase, post-log phase and stationary phase required 2% CO_2, 5% CO_2, and 16% CO_2, respectively.

These results obtained at pH 6.4 were not applicable at pH 6.7; at this pH the % CO_2 required for immediate logarithmic growth decreased one-half and at pH 7.0 the optimum % CO_2 again decreased by half, or 4 fold over all for a pH change from 6.4 to 7.0. The relative ratios of CO_2 needed for inocula of different physiological ages remained the same.

Equilibrium ratios between CO_2 and bicarbonate at a given pH is proportional to the concentration of CO_2. When the pH is increased 0.3 pH unit the bicarbonate ion concentration is doubled, and to return to the original bicarbonate concentration, the CO_2 must be reduced 50%. The correspondence between the CO_2 requirement for growth at various pH values and the physiochemical effect of CO_2 on bicarbonate ion concentration clearly demonstrated that H. eutropha required bicarbonate ion, not CO_2, during initial growth.

The ability to completely eliminate the lag period by any means is such a significant observation for cell growth and control that it was incumbent to determine if other types of cells were similarly affected by CO_2 or bicarbonate.

2) E. coli grown aerobically and Streptococcus group H (Challis) grown anaerobically both required CO_2 to initiate immediate growth on subculture. Streptococcus H, which was studied more intensively, was unlike H. eutropha in that CO_2 was the primary requirement, not bicarbonate. The CO_2 requirement was not significantly changed by a change of the pH of the medium.

Thus preliminary results show that CO_2 or bicarbonate play an unexpectedly important role in initiating growth in an otherwise complete medium.

3) The hydrogenase from H. eutropha has been purified 80 fold from cells with an 8 fold higher content of hydrogenase than normal cells. The highly active enzyme is reasonably stable when procedures developed to eliminate oxygen contamination are used. Several physical and biochemical characteristics of the enzyme are still to be determined before publication of the results.

4) The next sequential enzyme in the electron transport system is NADH-cytochrome c reductase. Purification of this enzyme has reached 7 fold.

Significance to Bio-Medical Research and the Program of the Institute:

A general requirement for CO_2 by Hydrogenomonas eutropha is established in the definition of an autotroph; the sharp optimum for CO_2 is of interest but has no special significance. However, for the first time it has been conclusively shown by simple experiments that bicarbonate, not CO_2, is the species of carbon used by H. eutropha. Of great interest is the observation that any nutrient (bicarbonate in these experiments) is capable of eliminating the normal period of adjustment. The test conditions used would have insured a lag period with any organism because the inoculum was small and was taken from a stationary phase culture which had been stored for a week.

The lag period is such a universal phenomenon that abridging this phase of growth in one organism by bicarbonate (CO_2) naturally raised the question whether bicarbonate (CO_2) had a similar effect on other organisms. It was shown with both an aerobic heterotroph, E. coli, and an anaerobic heterotroph, Streptococcus group H, that CO_2 had the same effect of eliminating the lag period. The spectrum of organisms tested thus far is still too limited to make definite conclusions; however, the consistency of the CO_2 effect on the lag period of the diverse organisms tested strongly suggests CO_2 or bicarbonate will have the same effect on most or all organisms.

Our demonstration that the lag period can be eliminated by the simple addition of CO_2, suggests that the 'normal' classical growth curve may be an artifact of the method of laboratory cultivation of microorganisms. The CO_2 requirement undoubtedly was missed because cultures eventually produce CO_2

endogenously, permitting growth to occur. CO_2 had remained a step-child in microbial nutrition except where CO_2 has been found to be required for cultivation of certain fresh isolates of pathogens (e.g. Neisseria and Brucella).

If the lag period can be eliminated in culture growth the increase in production and the saving in time would be considerable. Equally important would be the possibility that contaminants requiring less CO_2 could not obtain selective advantage during a long lag period. Of most importance is that more profound basic questions concerning initiation of cell growth can now be asked and tested experimentally.

Proposed Course of Project:

A) Culture Studies

Select bacteria representative of various types (aerobes, anaerobes, bacilli, cocci, autotrophs, simple nutrient heterotrophs and complex nutrient heterotrophs) to determine if CO_2 in some form is a general requirement for growth.

Use labeled CO_2 to determine the fate of CO_2. Is CO_2 fixed into a required compound or does it act as an initiator in some enzyme system?

B) Enzyme Studies

Purify NADH-cytochrome c reductase and determine its physical and biochemical characteristics.

Identify the enzyme system involved in CO_2 activation of growth.

Honors and Awards:

Presentation of invited paper "Bicarbonate ion requirements for determination of lag period in Hydrogenomonas eutropha", American Society for Microbiology National Meeting, symposium on autotrophy, May 6, 1971, Minneapolis.

Seminar, Howard University, Bacterial Electron Transport Systems, March 30, 1971.

Publications:

Repaske, R.: A Method for Anaerobic Column Chromatography. In Jakoby, W., (ed.): Methods in Enzymology. New York, New York, Academic Press, 1971, Vol. 22, pp. 322-325.

Repaske, R., Ambrose, C. A., Repaske, A. C. and De Lacy, M. L.: Bicarbonate Requirement for Elimination of the Lag Period of Hydrogenomonas eutropha. J. Bact. (in press).

Serial No. NIAID 62
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical Studies on Staphylococcal Cell Walls

Previous Serial Number: Same

Principal Investigator: Dr. Eskin Huff

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	-	-	24/12
Professional	12/12	-	-	12/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

We are interested in understanding the processes involved in the formation and breakdown of bacterial cell walls. The widespread occurrence of lytic and autolytic enzymes in bacteria and in all of the staphylococcal strains which we have examined, has led us to investigate the role of these enzymes in the bacterial cell physiology. We have been especially concerned with a) the intracellular location of these enzymes in Staphylococcus aureus, b) the mode of action of the enzyme on the cell wall, c) the mechanism by which the lytic enzymes of a cell are kept under control, and methods for chemically characterizing and quantitating cell wall components.

Methods Employed:

Growth of cells and preparation of cell walls is as has been previously described. New methods have been developed for the separation of cell wall hydrolysates into amino acid, neutral, and phosphate ester fractions followed by separation and quantitation on gas liquid chromatograph (GLC). The hydrochloric acid hydrolysate of cell wall material is passed through a Dowex 50 H^+ column (0.24 ml) in tandem with a Dowex I OH^- column. The neutral fraction which passes through is dried under a stream of filtered air at 37°. The phosphate esters are eluted from the Dowex I column with ammonium formate and lyophylized. The amino acids are eluted from the Dowex 50 with HCl and lyophylized over soda lime. The trimethylsilyl derivatives are prepared of neutral, phosphate ester and amino acid fractions using a mixture of N, O-Bis-(Trimethylsilyl)-Acetamide and trimethylchlorosilane (7:3). Since only a nanomole of compound is needed for quantitation on the GLC, a 2 to 3 milligram sample of cell wall gives a complete analysis.

Major Findings:

As a part of a continued study of the content of autolysins in S. aureus we have tested 19 additional strains for cell wall autolysis. Many of these strains were mutants derived from 968, a strain having an unusually high autolysin content. All of the strains derived from 968 had high autolysin content. The overall range in autolysin level for all strains examined was 56-fold. The stimulation of the autolysis by magnesium varied from no stimulation to 6-fold stimulation.

The cell walls of strain 968 are completely autolyzed in 10 minutes, but whole cells autolyze at only 1/10 this rate. In order to determine the nature of this autolysin we have autolyzed the cell walls of a variant of 968 (strain E) and attempted to characterize the autolysis products. Fractionation of the autolysate on a Sephadex G-50 column yielded two major phosphate-containing peaks. Material from each was hydrolyzed with acid and also with base. The first peak gave 8 spots on thin layer chromatography (TLC) after acid and 6 spots after base hydrolysis. The material from the second peak gave 3 spots on TLC after acid and 2 spots after base hydrolysis. One of the major spots moved on TLC the same as α-glycerol phosphate. The small amounts of material available for further chemical analysis combined with the complexity of the material led us to develop a procedure for identification of ribitol phosphate and glycerol phosphate by GLC of the trimethylsilyl derivatives with ethylene glycol phosphate as internal standard. Other methods developed were 1) a method for the determination of glycerol, ribitol, anhydroribitol, erythritol, propylene glycol and ethylene glycol using 2-deoxyribitol as an internal standard and GLC of the trimethylsilyl derivatives; 2) a method for the determination of the cell wall amino acids alanine, glycine, glutamic acid, aspartic acid, lysine, diamino pimelic, ornithine, serine, and threonine by GLC of the trimethylsilyl derivatives with valine as internal standard; 3) a method for the determination of leucine, methionine, norvaline, isoleucine, phenylalanine, α-aminobutyric acid, proline and cysteine by GLC of the trimethylsilyl derivatives using valine as internal standard.

Hydrolysis of the cell walls of Streptococcus Challis yields glycerol and 2 unknown neutral compounds. The major amino acids present are lysine, aspartic acid, glycine, and alanine with small amounts of serine and glutamic acid and some unidentified amino acids.

Significance to Bio-Medical Research and the Program of the Institute:

New, sensitive, and rapid techniques have been developed for the analysis of acidic, basic and neutral components of bacterial cell wall hydrolysates using ion exchange and gas-liquid chromatography. The ease with which this can be accomplished should facilitate structural analysis of bacterial cell walls and cell-wall-fragments released by lytic and autolytic enzymes. These methods are necessary for our study aimed at determining 1) why different strains of Streptococcus and Staphylococcus vary in their susceptibility to lytic enzymes and autolysins and 2) the mode of action of autolysins. Our studies on the localization, mechanisms of activation and control of autolytic enzymes in Staphylococcus aureus and Streptococcal species could lead to the preparation of drugs which would activate the native bacterial autolysins in these organisms causing them to self destruct. Such a drug might be valuable for the treatment of Staphylococcal or Streptococcal infections resistant to other forms of treatment.

Proposed Course of Project:

The comparison of S. aureus strains for autolysin level in the cell wall has been extended. We propose to compare chemical differences in closely related high and low autolysin producers to see if there are structural differences which influence the rate of autolysis of a cell wall. We also wish to see if there are differences in the type of autolysin and content of autolysin. Ellwood and Tempest (Proc. Soc, Gen. Microbiol. Aug. 1969) reported that when Staphylococcus aureus H is grown in a magnesium limited environment in a chemostat it has ribitol teichoic acid in the cell wall with 3% phosphorus. When they were grown in a phosphate limited environment the ribitol teichoic acid was replaced with teichuronic acid. The phosphate level in the cell wall fell to 0.4%. We wish to grow S. aureus 968 under conditions of high and low phosphate to see if we can show a shift from teichoic acid to teichuronic acid and if so what effect this has on the autolysin content of the cell wall.

Using the sensitive gas-liquid chromatography procedure which we have recently developed we wish to survey the Staphylococci and Streptococci to see if we can observe any unusual cell wall components.

Honors and Awards:

None.

Publications:

Huff, E., Silverman, C. S., Adams, N. J., and Awkard, W. S.: An extracellular cell-wall lytic enzyme from Staphylococcus aureus. Purification and partial characterization. J. Bact. 103: 761-769, 1970.

Serial No. NIAID-63A
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Occurrence, Properties and Significance of Protein Iron-Chelators in the Biochemistry of the Acquisition of Iron, and Possibly Other Metals, by Microbial and Animal Cells.

Previous Serial Number: Same

Principal Investigator: Dr. Arthur L. Schade

Other Investigators: None

Cooperating Units: Laboratory of Clinical Investigation, NIAID (NIAID-33(C))

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1/12	-	-	1/12
Professional	1/12	-	-	1/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the occurrence, properties, and significance of protein iron-chelators and to investigate the mechanisms by which microbial and animal cells acquire from plasma siderophilin, ekkrinosiderophilin, and conalbumin the iron, and possibly other metals, for normal growth and function and the effect of physiological conditions upon this exchange.

Methods Employed:

Hematological procedures, radioactive tracer techniques, manometric techniques, strip and disc electrophoresis, chromatography, optical rotatory dispersion, infrared spectroscopy, sucrose gradient sedimentation, and immunologic analyses.

Major Findings:

The growth of Candida albicans in media containing ekkrinosiderophilin (lactoferrin) was investigated. Human milk, with an unsaturated iron-binding capacity of approximately 6 µg Fe/ml, served as the primary ekkrinosiderophilin-rich medium for these studies. The results showed that the iron-free protein markedly impaired replication of the yeast. The growth inhibitory property was lost when the iron-binding protein was saturated with iron. The findings suggest that iron-chelating agents may have clinical application in treating disorders such as chronic superficial candidiasis.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of protein-iron chelators in biological fluids which have contact with the exterior environment as well as in those providing the intimate milieu of host organs and their constituent cells is a regulatory factor in the iron, and possibly other metal, nutrition and metabolism of both host and parasite, and consequently in their mutual relationships. In addition to determining where and in what concentrations such chelators are found in biological fluids, it is desirable to establish the conditions governing the availability of the metal and the mechanism of its transfer to host tissues and to the offending parasite. The results of such investigations involving nutrilites, such as iron, so essential to the life and metabolism of both host and parasite, should increase our understanding of infection and its control.

Proposed Course of Project:

We propose to investigate the bacterial growth-inhibiting properties of ekkrinosiderophilin of human milk and its probable significance to establishment of the bacterial flora of the exclusively milk-fed infant.

Honors and Awards:

Member Scientific Advisory Committee, Annual Colloquium, Protides of the Biological Fluids, Brugge, Belgium.

Publications:

Schade, A. L. and Reinhart, R. W.: Galactothermin - a reversibly heat-precipitable protein of human milk at neutral pH. Biochem. J. Vol. 118: 181-186, 1970.

Serial No. NIAID 63B
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Nutrition, Physiology and Ultrastructure of Staphylococcus aureus.

Previous Serial Number: Same

Principal Investigator: Dr. T. S. Theodore

Other Investigators: Dr. Roger M. Cole and Mr. Terry Popkin

Cooperating Units: Dr. Charles Panos, Albert Einstein Medical Center, Philadelphia, Pa.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	18/12	-	-	18/12
Professional	9/12	-	-	9/12
Other	9/12	-	-	9/12

Project Description:

Objectives:

To isolate and characterize by cytological and biochemical methods the membrane system of Staphylococcus aureus; more specifically, to examine the distribution of certain enzymes and compounds in the membrane system in order to allow for the physiological, as well as histochemical differentiation of mesosomes and plasma membrane.

Methods Employed:

Routine bacterial and cultural procedures and specialized procedures for the preparation of de-ironized media; spectrophotometry, chromatography, electrophoresis and routine metabolic, enzymatic and radioisotope procedures; differential and gradient centrifugation techniques and electron microscopy.

Major Findings:

Our investigations this past year have been directed towards the chemical characterization of plasma membranes and mesosomal vesicles isolated from S. aureus ATCC 6538P by methods previously reported (Project 63B, 1969-70). The fractions used in these studies were obtained from upper bands of sucrose gradients and were shown by electron microscopy to be clean and homogeneous preparations.

A. Gross chemical composition of membranes and mesosomes.

The gross chemical composition of plasma and mesosomal membranes is shown below:

	% protein	% lipid	% RNA	% carbohydrate
membranes	56	25	14	4
mesosomes	41	34	8	4

The plasma and mesosomal membranes were chemically similar, being composed chiefly of lipid and protein which together account for 75-80% of their dry weights. Smaller amounts of carbohydrate and RNA were also present. The latter was twice as high in the plasma membrane fraction where membrane-bound ribosomes might be expected and in which electron micrographs showed membranes not completely devoid of ribosomes. No DNA was detected in any of our fractions after DNase treatment.

B. Protein, lipid and fatty acid composition of membranes and mesosomes.

Using polyacrylamide gel electrophoresis, we showed differences in protein composition between membranes and mesosomes. Membrane and mesosomal proteins were solubilized in phenol-acetic acid-urea and separated on acid gels. Both membrane fractions had approximately 25 different protein bands; however, the mesosomal gel patterns were distinctly different, both qualitatively and quantitatively, from the membrane patterns. At present, we do not know whether these differences are associated with structural proteins or (and) functional proteins (enzymes) of the membranes and mesosomes. We are now attempting to characterize these protein differences by gel electrophoresis using selective protein staining techniques (i.e.; glycoproteins, lipoproteins, ribonucleoproteins and iron-containing proteins) and by enzymatic analysis.

Qualitatively, no marked differences in lipids or fatty acids were found between the membranes and mesosomes. Lipids, extracted with chloroform-methanol and analyzed by thin layer chromatography, consisted mostly of phosphatidyl glycerol, cardiolipin, 2 glycolipids, vitamin K_2, and an amino acid-containing phospholipid. Fatty acids, analyzed by gas-liquid chromatography (in collaboration with Dr. C. Panos, Albert Einstein Medical Center), consisted of normal, iso and anteiso, saturated fatty acids of from 10-20 carbon lengths.

Presently, we are doing large scale fractionation of membranes and mesosomes in order to obtain sufficient amounts of lipid material for fractionation and quantitation. Also, we are especially interested in determining to what extent C_{55} isoprenoid alcohols (membrane bound lipid intermediates involved in cell wall biosynthesis) are present in our membrane fractions.

C. Cytochemical localization of respiratory and chemical activities of the cell.

Attempts to show cytochemical localization of respiratory and chemical activities in S. aureus; especially in association with the membrane fractions, has not been very successful. We have tried to localize succinic dehydrogenase using tetranitroblue tetrazolium, iron using potassium ferricyanide reduction, and oxidative activities using tellurite reduction. Of the three techniques, tellurite reduction appears to be the most promising and is now under investigation

Significance to Bio-Medical Research and the Program of the Institute:

Our studies continue to be directed toward a basic understanding of the structural and functional interrelationships between membranes and mesosomes of S. aureus. The approach has been to isolate these organelles in a relatively pure and intact state and to characterize them by chemical and enzymatic analysis. We found no significant differences in gross chemical composition between membranes and mesosomes; however, electrophoretic analysis of their proteins on polyacrylamide gels revealed both quantitative and qualitative differences. This is of considerable interest since it is the first time we were able to show any clear-cut distinction between membranes and mesosomes. Currently, we are in the process of identifying these protein differences. It is anticipated that such studies will enable us to ascertain what specific functions reside in these organelles and how they are related to the cell's economy.

Proposed Course of Project:

Large scale fractionation of membranes and mesosomes are underway and the following lines of investigation will be pursued:

1) Ultrastructure characterization of isolated membranes and mesosomes by freeze-etching technique and continued attempts at cytochemical localization of respiratory activities.

2) Detailed chemical characterization of the proteins, lipids and fatty acids.

3) Enzymatic analysis pertaining to cytochrome biosynthesis, lipid synthesis and enzymes involved in intermediary metabolism.

Honors and Awards:

Invited to submit paper on, "The separation and isolation of plasma membranes and mesosomal vesicles from Staphylococcus aureus," to new international journal, "Preparative Biochemistry."

Publications:

Theodore, T. S., Popkin, T. J., and Cole, R. M.: The separation and isolation of plasma membranes and mesosomal vesicles of *Staphylococcus aureus*. *Preparative Biochemistry*, 1, No. 3, 1971 (in press).

Ranhand, J. M., Theodore, T. S., and Cole, R. M.: Protein difference between competent and noncompetent cultures of a group H streptococcus. *J. Bact*. 104: 360-362, 1970.

Serial No. NIAID-63C
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Investigations of Gram-Positive Cocci

Previous Serial Number: Same

Principal Investigator: Dr. Arthur L. Schade

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	27/12	-	-	27/12
Professional	11/12	-	-	11/12
Other	16/12	-	-	16/12

Project Description:

Objectives:

A) To explore the growth of group A streptococci in various blood sera or plasmas, in order to establish a baseline for correlation for function and structure.

B) To investigate conditions for protoplast formation in strains of streptococcus A and the release of their mesosomes for subsequent study of isolated mesosomal structure and function.

C) To investigate the protein composition of streptococcal A plasma membranes and mesosomes for identity or non-identity of their structure and function.

Methods Employed:

Standard methods of bacteriology, metabolism, and biochemistry. Special methods as required including chemical analyses for DNA, radioactivity techniques, spectrophotometry, electrophoresis, chromatography, and ultracentrifugal gradient separations.

Major Findings:

A) Growth of Streptococci in Human Serum.

Following the observation that our type 4, M protein-negative strain of Streptococcus A grows poorly in 100% human serum as compared to 100% rabbit serum as culture media, we extended our investigations to strains of type 1, 4, and 6 carrying M protein. Human serum inhibited growth of all these strains. Our previous finding that veal infusion alone, of a variety of usual complex growth media tested, was effective in completely overcoming the growth inhibitory property of human serum deserves further close investigation. Identification of the basis for the observed inhibition might rationally lead to means of enhancing such inhibition in vivo.

B) Protoplast Formation and Isolation of Plasma Membranes and Mesosomes.

The use of sucrose as a stabilizing agent in the production of protoplasts from the Streptococcus A, type 4 strain, even at one molar concentration, led to formation of sufficient acid to lower the pH of the protoplasting medium to pH's of 5.30 - 5.40 although initial pH values varied from 6.0 to 7.8 in 0.05-M tris-maleate buffer. Anaerobic glycolytic studies of this Streptococcus with galactose, levulose, glucose, melibiose, sucrose, and raffinose showed acid production only from glucose, sucrose, and levulose. Respiration by this organism was limited to the same three sugars. These results recommend the use of either raffinose or triglycine as stabilizing agent. However, triglycine at 0.90 osmolality did not maintain protoplast integrity. Raffinose at 30% concentration (w/v), osmolality = 0.737, in a tris-maleate buffer at 0.05-M concentration plus $MgCl_2$ at 0.025-M over a pH range of 5.1 to 7.6 with Streptococcus C phage lysin digestion at 37°C for 40 minutes provided good protection to formed protoplasts with insignificant changes from the initial pH values and no loss of DNA to the supernatant. pH's of 5.1, 5.6, and 7.6 retarded lysin activity while over the pH range of 6.0 - 7.0 maximal wall lysis occurred. At pH 7.6 some magnesium precipitated. We conclude that with raffinose as stabilizer, a pH range of 6.2 - 6.4 is very satisfactory for the lysin activity and protoplast formation.

By determining the loss in turbidity at λ600nm of protoplasting cell suspensions in 30% raffinose at pH 6.3, we assessed the effect of concentrations of magnesium varying from zero to 0.1-M on the maintenance of protoplast integrity during their production over a 30 minute wall digestion period. The nearer 0.1 molar concentration of magnesium was approached, the better was the suspension's turbidity maintained. Taking protoplast suspensions produced in 30% raffinose with 0.001, 0.025, and 0.1-M magnesium and kept for 20 hours at room temperature, we diluted them 1:55 in aqueous $MgCl_2$ solutions ranging from 0 to 0.1-M concentration and followed turbidity values at λ600nm. Only when the concentration of magnesium was lower than 0.01-M was there a precipitous fall in the turbidity of the diluted protoplast suspensions. For the purpose of assuring the formation of stable protoplasts from our Streptococcus A strain, we settled on the use of 30% raffinose buffered at pH 6.2 - 6.4 and supplemented with 0.01 to 0.1 molar magnesium with preference given to the higher concentrations. After such a protoplasting procedure, mesosomes were separated from the wall digest supernatants by centrifugation and purified on sucrose gradients while plasma membranes were prepared by osmotic shock of the precipitated protoplasts. The purified mesosomes and exhaustively washed membranes were analyzed for their carbohydrate, nucleic acid, lipid and protein composition.

C) Electrophoretic Analyses of Extracted Proteins from Whole Cells, Plasma Membranes, and Mesosomes.

With phenol-acetic acid mixture (P-A) as protein extractant, cells obtained from cultures over a 48 hour growth period at 37° in trypticase-soy broth fortified with yeast extract, glucose, and cysteine were extracted in the fresh wet state, after lyophilization, and following muralysin treatment to dissolve the cell walls. Turbidity-growth curves showed no growth lag of inoculum cells from an overnight culture. Log growth continued up to 5-1/2 hours incubation, followed by progressive fall in growth rate to some reduction in culture turbidity at 24 hours. Cells from zero-time, 1/2 hr., 1-1/2 hrs., 3-1/2 hrs., 5 hrs., 7 hrs., 9-1/2hrs., 25-1/2 hrs., and 48 hrs. were analyzed on acid polyacrylamide gels. No qualitative differences were found between the protein band patterns from cells at any stage of growth when the whole cells, fresh or lyophilized, were analyzed. No qualitative differences were found in band patterns from the muralysin-treated cells of any age. However, between whole cell extracts and extracts from lysin-treated cells of the same age dramatic quantitative differences were observed. Kjeldahl nitrogen analyses of the two types of extracts confirmed the disparity of their nitrogen concentrations. Qualitative differences observed might be referable to the quantitative disparities. By running protein standards of known molecular weight, it was determined that proteins above weights of 15-20,000 were extracted by P-A with great difficulty when the cell wall was intact. Extension of extraction time up to 6 hours at 37° did not significantly add to the quantity of the higher molecular weight proteins extracted. Fifteen proteins were delineate from P-A extracts of whole cells while 24 were seen from P-A extracts of muralysin-treated whole cells.

P-A extracts of purified plasma membranes when acid gel electrophoresed exhibited 24 protein bands while extracts of sucrose gradient separated mesosomes yielded 20 protein bands. The band patterns were qualitatively and quantitatively different suggesting some unique protein composition for each organelle. Confirmatory work is required and in progress to establish these indicated differences. Qualitative lipid determinations on these two structures showed no significant differences.

By use of a recently reported procedure employing 10% mercaptoethanol, Triton, urea, and potassium carbonate, whole cells as well as muralysin-treated cells were extracted at alkaline pH and then electrophoresed on a discontinuous acid pH gel. As many as 33 well banded proteins were visualized by staining of the electrophoresed muralysin-treated extract while only 8 bands were clearly visible from the extract of the whole cells. The high molecular weight species of proteins were again virtually absent in the latter case. Application of the Schiff staining technique for demonstrating glycoproteins in a polyacrylamide gel was made to a gel containing the separated proteins from the extract of the muralysin-treated cells. Six glycoprotein red-stained bands appeared, all near the beginning of the separating gel where the higher molecular weight proteins are expected to be found.

Significance to Bio-Medical Research and the Program of the Institute:

The bacteriostatic action of human serum on group A streptococci warrants investigation for reasons of immune response and protection against this human pathogen.

Investigation of the structure and metabolic activities of protoplasts, plasma membranes and mesosomes of the streptococci will contribute to our knowledge of the function of such components in the cell's economy and provide a necessary basis for evaluating any structural anomaly that may be correlated with a functional anomaly.

Since the major constituents of both the plasma membranes and mesosomes are proteins with both catalytic and structural properties it is essential to our understanding of the functioning of these organelles that they be identified and characterized. Of unique and prime importance for the life and growth of any cell is its possession of a membrane upon whose bounding character its individuality depends and across which its necessary contact with the environment is maintained and regulated. To determine the characteristics of the proteins peculiar to membrane assemblage and dynamic persistence in a commonly solubilizing aqueous milieu is to elucidate a primary basis for the dependence of function upon structure in biology.

Proposed Course of Project:

Investigations of the protein composition of plasma membranes and mesosomes of Streptococcus group A organisms to establish their intimate structural identity or non-identity and to relate, where possible, such structure to their cellular function, will continue. Emphasis will be put upon studies to elucidate the features peculiar to the streptococcal membrane proteins favoring their assemblage, in concert with lipids, into an aqueous insoluble membranous organelle. As time and technical assistance permits, studies will be continued to determine the characteristics of the indicated bacteriostatic factor in human serum that appears to be specific for group A streptococci and to isolate it for definitive chemical description.

Honors and Awards:

Continuing membership: U.S. Civil Service Board, Microbiology Panel. Vice-Chairman - Advisory Board, Service and Supply Fund, NIH.

Publications:

None.

Serial No. NIAID-64A
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies with synthetic potential antibacterial, antifungal, antiparasitic, and antitumor agents.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: Mr. George Caldes

Cooperating Units:
Section on Medical Mycology, LM (NIAID-78A)
Dr. George Lones

Cancer Chemotherapy National Service Center, NCI

Dr. Leslie Eidus
National Reference Center for Tuberculosis
Dept. of National Health and Welfare
Ottawa, Canada

Dr. K. I. Melville (Professor)
Dept. of Pharmacology & Therapeutics
McGill University
Montreal, Canada

Dr. Sergio Rabinovich
Dept. of Medicine
Division of Infectious Diseases
University of Iowa
Ames, Iowa

Dr. Leo Rane
University of Miami Medical School
Miami, Florida

Dr. Barrie R. Jones
(Director of Clinical Ophthalmology)
Institute of Ophthalmology
University of London
London, England

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	8/12	-	-	8/12
Professional	4/12	-	-	4/12
Other	4/12	-	-	4/12

Project Description:

Objectives:

Further studies on the synthesis of new series of compounds as potential non-toxic chemotherapeutic agents against bacterial, fungal, parasitic infections had as anticancer agents.

Methods Employed:

A series of 25 new sulfones were prepared by the condensation of molecular amounts of dichlorodiphenylsulfone with mercaptans in methylcellusolve in the presence of sodium hydroxide to neutralize excess hydrochloric acid formed in the reaction. The mixture was refluxed on a hot plate for 2 hours. The soluble reaction mixture was neutralized to pH 5.0 with sodium hydroxide and precipitated with cold water. The products obtained were crystallized from 70 percent methanol and the final products analyzed for carbon, hydrogen and nitrogen, and melting points determined. Twenty new compounds were synthesized by reacting sulfinic acid in 50 percent ethanol with aromatic aldehydes under reflux. The final crystalline products were also analyzed for elemental carbon, hydrogen and nitrogen. A series of 30 p-fluorophenylthiosemicarbazones were prepared by first reacting p-fluorophenylisothiocyanate with hydrazine to form the thiosemicarbazide. The thiosemicarbazide was condensed with alphatic, aromatic and heterocyclic aldehyde to produce yellow to orange crystalline thiosemicarbazones. Since cycloserine deteriorates rapidly in heat, the compound was condensed with aldehydes to produce Schiff bases which are more stable and resistant to heat (25 derivatives).

Major Findings:

Antifungal Activity. Forty-six furfurylazines and 50 hydrazones of p-methoxybenzylhydrazino-carboxylate (synthesis reported in previous project reports) were tested for in vitro antifungal activity against Histoplasma capsulatum and Cryptococcus neoformans. The procedure for testing the compound was described in previous reports. In the furfurylazine series, the 3,5-dichlorosalicylaldehyde derivative possessed in vitro antifungal activity against H. capsulatum in a dose of 20 μg/ml. The salicylaldehyde, o-chlorobenzaldehyde, p-nitrobenzaldehyde, piperonal, 3-iodobenzaldehyde, 3,5-diiodo-2-hydroxybenzaldehyde, 4-chloro-3-nitrobenzaldehyde, 3-benzyloxybenzaldehyde, 3,4-dichlorobenzaldehyde, and 1-naphthaldehyde inhibited growth of H. capsulatum in broth in a concentration of 100 μg/ml for 72 hours. The compounds were less active against C. neoformans. Only the 3,5-diiodo-2-hydroxybenzaldehyde inhibited growth at 100 μg/ml while compounds salicylaldehyde, 3,5-diiodo-2-hydroxybenzaldehyde, and 5-bromoindole-3-carboxaldehyde inhibited growth at 500 μg/ml. Among the 50 hydrazones of p-methoxybenzylhydrazinocarboxylate, only the 3-bromobenzaldehyde and 3,5-dimethyl-4-hydroxybenzaldehyde derivatives inhibited the growth of H. capsulatum at 100 μg/ml while 16 derivatives were active in a 500 μg/ml concentration. The latter compounds showed very little in vitro antifungal activity against C. neoformans.

Antimycobacterial Activity. The new sulfone derivatives are being studied as antimycobacterial agents against isoniazid sensitive and resistant strains of M. tuberculosis by Dr. K. Melville and Dr. L. Eidus. The cycloserine derivatives are also being tested for chemotherapeutic activity and stability to heat against M. tuberculosis.

Other Activities. Dr. Sergio Rabinovich at the University of Iowa is studying a series of 4-(1-adamantyl)-3-thiosemicarbazones as potential chemotherapeutic agents against antiviral H. influenzae type B in experimentally infected animals. Synthesis of chalcone derivatives, reported in last year's project report, have been tested in vivo as antitumor agents. The β-methylchalcone derivative of 1,4-dihydrazinophthalazine has been shown to possess antitumor activity against Leukemia L-1210 and the Walker carcinosarcoma 256 intramuscular tumor in animals. The chalcone (dypnone) derivative of guanylhydrazone was found to be effective as an antimalarial agent in mice experimentally infected with Plasmodium berghei.

Significance to Bio-Medical Research and the Program of the Institute:

There is still a need for synthesis of numerous non-toxic organic compounds with potentially high prophylactic and chemotherapeutic activity in relatively low concentration against any of the pathogenic agents which cause infection in humans. Toxicity of a drug and resistance of the infecting agent to a chemotherapeutic agent used over prolonged periods limits the usefulness of the agent. With non-toxic compounds, large doses may be administered. Higher blood levels might thus be achieved which may have some effect on therapy and the prevention of the appearance of resistant forms of the infecting agent. Certain compounds synthesized in this laboratory have been shown to exhibit consistent chemotherapeutic activity against experimental infection (P. berghei) and antitumor activity in animals with no or undetectable macroscopic toxicity. These compounds may be useful as chemotherapeutic agents against human malarial infections and as antitumor agents.

Proposed Course of Project:

This project will be terminated during the year in order to devote most of our time to more relevant problems of the Laboratory of Microbiology, especially those concerned with bacterial membrane studies.

Honors and Awards:

Invited to attend and participate at the 5th International Symposium on Carbohydrate Chemistry. Paris, France, Aug. 17-22, 1970.

Toxicology Information Program (TIP) at the National Library of Medicine included my name and qualifications in the "Roster of Authorities in Specialized Subtopics in Toxicology." October, 1970.

Invited to attend and participate at a Symposium in Organic Chemistry. Louisiana State University, Baton Rouge, Louisiana. Feb. 22, 1971.

Invited to attend and present a paper entitled, "Protective effect of certain sulfonic acids on the toxicity of lucanthone," at the 7th Annual Meeting of the Canadian Society of Chemotherapy, Magog, Quebec, Canada.

Consultant to Vaccine Development Committee, Infectious Disease Branch, Collaborative Research Division, NIAID.

Publications:

Prescott, B.: Potential antimalarial agents. III. Derivatives of 4',4'''-sulfonylbis(3-Chloropropionanilide). Internat. J. Clin. Pharmacology, Therapy and Toxicology. (in press)

Prescott, B., Lones, G., Peacock, C., and Caldes, G.: 4-(1-adamantyl)-3-thiosemicarbazones as potential chemotherapeutic agents. Antimicrobial Agents and Chemotherapy. Bethesda, Md., American Society for Microbiology, 1969, pp. 275-279.

Serial No. NIAID-64C
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Further studies on the preparation, antigenic properties, and elucidation of the structure of a serologically active glycolipid hapten from *Mycoplasma pneumoniae*.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: Dr. Roger M. Cole
Mr. George Caldes
Mr. Terry Popkin

Cooperating Units: Laboratory of Infectious Diseases (NIAID-70C)
Dr. Robert M. Chanock
Dr. Shmuel Razin (Visiting scientist, Hadassah Medical School, Jerusalem)
Mr. Walter James

Laboratory of Arthritis and Metabolic Diseases
Dr. John L. Decker

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	10/12	-	-	10/12
Professional	4/12	-	-	4/12
Other	6/12	-	-	6/12

Project Description:

Objectives:

Further production and purification of a serologically active glycolipid hapten from *M. pneumoniae*.

Methods Employed:

The methods used for the isolation and fractionation of the serologically active hapten have been described in previous reports. Until an abundant source of *M. pneumoniae* active glycolipid becomes available, no vaccination with reaggregates can be planned. Thus, large quantities of *M. pneumoniae* lipids were fractionated by DEAE-cellulose and silicic acid chromatography for the production of the serologically active glycolipid hapten. The material was tested for purity of glycolipid by TLC chromatography and analytical analysis, since a small amount of phosphatidylglycerol accompanies the glycolipids in the previous procedures for purification. The active subfractions of the lipid hapten from silicic acid chromatography were pooled and again rechromatographed on columns of DEAE-cellulose and the chloroform-methanol (7:3) fraction was chromatographed on TLC plates. The material was a mixture and produced 6 glycolipid spots on the plates and no detectable phospholipids or phosphorus by analytical studies. Carbohydrate was determined by hydrolyzing samples of purified glycolipid. Reaggregation consisted of the solubilization of lipid-depleted *A. laidlawii* membranes and *M. pneumoniae* glycolipids in 20 mm sodium dodecyl sulfate and dialysis of the mixed solutions against 20 mm Mg^{2+} for 4 days in the cold (4°C). The material in the sac was centrifuged at high speed and the pellet redissolved in deionized water. The reaggregate was tested for RNA, DNA, carbohydrate, and lipid.

Major Findings:

The studies show that *M. pneumoniae* glycolipids become immunogenic when bound to *A. laidlawii* membrane proteins by the reaggregation process. Chromatographic behavior and serological activity of the incorporated glycolipids were not affected by the reaggregation process. Thin layer chromatography on the lipid before and after reaggregation gave similar patterns and the titer of the complement fixation test was essentially the same for both preparations. A close association with membrane proteins is apparently essential for optimal immunogenicity of membrane lipids. Mixing glycolipids and lipid-depleted membranes of *A. laidlawii* was inferior, immunogenically, to the hybrid reaggregate of the same components.

Fractionation of *Mycoplasma hyorhinis* for Dr. Decker, by methods described previously again, showed the major serologically reactive material (complement-fixation and growth inhibition tests) to be protein in nature. All lipid, carbohydrate and protein fractions were tried as skin test antigens in infected animals. The proteins isolated by chromatography of a sonicate soluble fraction of the defatted organisms on Sephadex G25 gave a strong positive skin reaction. Lipid and carbohydrate were negative in skin tests. Purification and characterization of the antigenic protein is in progress.

Significance to Bio-Medical Research and the Program of the Institute:

The advantage in using hybrid reaggregates for the production of antibodies to membrane lipids lies in the ability to select the lipid and protein components of the reaggregate and thus control the type of antibodies produced. *A. laidlawii* membrane protein is not related serologically to membrane protein of *M. pneumoniae*. Thus, the antibodies produced will not react with any of the *M. pneumoniae* cell components. *A. laidlawii* is a potential source for large quantities of essentailly pure membranes, which can be easily obtained by osmotic lysis of the fast growing organism, if a vaccine against M. pneumoniae based on the hybrid reaggregate principle is considered. In addition, A. laidlawii is rarely found in man and can be grown in the absence of serum, thus reducing problems of sensitization to vaccine components.

Proposed Course of Project:

Attempts to vaccinate against the important human respiratory pathogens with whole cell vaccines have not produced protection in animals, even though severity and duration of disease was reduced. All studies to date have been with such vaccines. Since the glycolipid hapten of M. pneumoniae requires a protein carrier to be immunogenic, experiments are currently in progress to reaggregate the purified hapten with lipid-depleted membrane protein of Acholeplasma laidlawii in a form acceptable for parenteral injection of humans. Whether vaccination with reaggregates of A. laidlawii protein with M. pneumoniae glycolipids is feasible insofar as protection against disease, is not yet known. The reaggregates are being tested in hamsters. If the results are encouraging, the methods can be applied to the preparation of a vaccine useful in man against M. pneumoniae.

Honors and Awards:

None

Publications:

Prescott, B., Chernick, S. S., James, W. D., Caldes, G., Barker, D., Sloan, H. R., and Chanock, R. M.: Mycoplasma pneumoniae phosphatidyl glycerol. Proc. Soc. Exp. Biol. Med. 134: 711-719, 1970.

Razin, S., Prescott, B., Caldes, G., James, W. D., and Chanock, R. M.: Role of glycolipids and phosphatidyl glycerol in the serological activity of Mycoplasma pneumoniae. Infection and Immunity 1: 408-416, 1970.

Razin, S. Prescott, B., and Chanock, R. M.: Immunogenicity of Mycoplasma pneumoniae glycolipids. Proc. Nat. Acad. Sci. 67: 590-597, 1970.

Razin, S., Prescott, B., James, W. D., Caldes, G., Valdesuso, J. R., and Chanock, R. M.: Production and properties of antisera to membrane glycolipids of Mycoplasma pneumoniae. Infection and Immunity 3: 420-423, 1971.

Cole, R. M., Popkin, T. J., Prescott, B., Chanock, R. M., and Razin, S.: Electron microscopy of solubilized Acholeplasma laidlawii membrane proteins reaggregated with Mycoplasma pneumoniae glycolipids. Biochim. Biophys. Acta 233: 76-83, 1971.

Serial No. NIAID-64D
1. Microbiology
2. Bacterial Structure
and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation, separation, and characterization of antigenic lipid hapten(s) from streptococci.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators:
Dr. James R. King
Dr. Roger M. Cole
Dr. Susan Birnbaum
Mr. George Caldes
Mr. Richard S. Whitt

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	27/12	-	-	27/12
Professional	15/12	-	-	15/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

Continuation of studies to isolate, purify, characterize, and test the lipid hapten (s) from Groups A and D streptococci responsible for the serological activity in the complement-fixation tests with Groups A and D rabbit antisera.

Methods Employed:

In a previous report, the isolation of a lipid hapten (s) from Groups A and D streptococci was described. This material was shown to react with complement-fixing antibodies. Briefly, the method for extraction of the lipids was as follows: Chloroform-methanol (CM) 2:1 extracts of the organisms were freed of non-lipid contaminants on columns of Sephadex G-25 fine and then resolved on columns of DEAE-cellulose by elution with a series of chloroform-methanol mixtures in varying ratios. In addition to the strains of streptococci described in last year's report, the following organisms were also studied for their lipid composition and serological activity of their lipid fractions in a similar manner: Group A streptococcus 60x298 L-form (type 13); Group A streptococcus ADA-L-form (type 14); Group H streptococcus strain Challis, Proteus mirabilis L-9; and Staphylococcus aureus (ATCC 23832).

In the experiments with Group A and D streptococci (parent and L forms) there was a similarity in the distribution of complement-fixing activity in the eluates from DEAE-cellulose chromatography. Since the DEAE-cellulose CM (7:3) eluate contained most of the material active in complement-fixation and was shown to be glycolipid in nature on thin layer chromatographic plates (TLC) with impregnated borate, most of our studies were carried out with this type of eluate. Thin layer chromatography and analytical studies have indicated that the DEAE-cellulose CM (7:3) fraction consisted of a mixture of glycolipids accompanied by a small amount of phosphatidyl glycerol. Samples of the active hapten were hydrolyzed for carbohydrate content with $2N\text{-}H_2SO_4$ in sealed tubes heated at 110°C for 2 hours. Acid was removed by weak base ion-exchange resin (Amberlite IR45, acetate form). The eluate was lyophilized, chromatographed on Whatman No. 1 paper and carbohydrate detected with aniline hydrogen phthalate and quantitated spectrophotometrically after elution of spots from paper.

Major Findings:

Rechromatography of the CM (7:3) fraction on DEAE-cellulose removed the minor amount of phospholipid present in the first CM (7:3) eluted fraction from DEAE. The final product showed no detectable phospholipids on TLC plates sprayed with the Dittmer reagent and no detectable phosphorus by the analytical procedure of Bartlett. Hydrolysates of the purified lipid haptens showed only glucose as the carbohydrate component detected in the serologically active glycolipid, either by the enzyme glucostat method or by paper chromatography. The optical activity of the glycolipid (CM 7:3) was found to be $[\alpha]_D^{20} + 59$.

Groups A and D streptococci may have more than one glycolipid hapten. Studies on the DEAE-cellulose CM (7:3) fraction show that this material was resolved into 5 components on TLC chromatoplates. When the 5 components were separated from the TLC plates, the major serologically, highly active material was recovered in 3 of the glycolipids while two of the fractions exhibited less CF activity. While TLC studies allow some assessment to be made of the heterogeneity of the CM (7:3) fraction, they do not permit easy separation and large scale purification of the individual lipid hapten components afforded by chromatographic techniques.

Antigenicity. The lipid hapten(s) CM (7:3) of Group A cross reacts with Group D rabbit antisera in the complement-fixation test and Group D lipid hapten with Group A rabbit antisera. The Groups A and D streptococcal lipid haptens do not react in the complement-fixation test with rabbit antisera to the following organisms: Group C streptococci; Group H streptococci, Challis strain; *Proteus mirabilis* L-9, *Staphylococcus aureus* ATCC-L; and *Mycoplasma pneumoniae* FH strain. In accord, the lipids from the latter organisms do not react in the complement-fixation test with rabbit antisera to Group A and D streptococci. Thus, the lipid hapten(s) appear common to Groups A and D streptococci but are serologically inactive in the complement-fixation test with antibodies to the other organisms studied.

Analytical Studies. Deacylation with mild alkaline hydrolysis or periodate treatment of the serologically active glycolipid destroys the complement-fixation activity, indicating that the activity may be related to stereospecificity of the molecular configuration of the hapten. Preliminary chemical characterization of the hapten indicated that the glycolipid contains glycerol, fatty acids, and carbohydrate components.

Significance to Bio-Medical Research and the Program of the Institute:

The demonstration of lipid haptens common to some different streptococci and their L forms, but not to others nor to other bacteria, raises interesting questions of taxonomic and serologic specificities among membrane antigens. The role of these bacterial glycolipids, other than as antigenic determinants, in unknown. Separation and complete chemical and physical identification of the several glycolipids may lead to useful information on membrane structure, and permit synthetic and reaggregation studies of value in clarifying the immunogenicity of streptococcal membranes.

Proposed Course of Project:

In order to perform additional analytical (glycerol-fatty acid-carbohydrate ratio) and *in vitro* and *in vivo* biological studies with purified serologically active glycolipid (s), preparation of large amounts of the DEAE-cellulose CM (7:3) fraction from Groups A and D streptococci (parent and L forms) will be carried out for subfractionation on silicic acid. To date, concentration of highly purified active lipid(s) by subfractionation on silicic acid has yielded very small amounts of material.

Honors and Awards:

None

Publications:

None

Serial No. NIAID-65
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electron microscopy of bacteria in relating structure and function.

Previous Serial Number: Same

Principal Investigator: Dr. Roger M. Cole

Other Investigators: Mr. Terry J. Popkin
Dr. Shmuel Razin (Visiting Scientist, LID, LM)

Cooperating Units: University of Arizona, College of Liberal Arts, Dept. of Microbiology and Medical Technology, Tucson (Dr. Neil Mendelson)

University of Cincinnati, College of Medicine, Dept. of Microbiology (Dr. J. Gabriel Michael)

State University of New York, Downstate Medical Center, Dept. of Medicine, Infectious Diseases Section, Brooklyn (Dr. Stephen J. Seligman)

University of California at Davis, Dept. of Microbiology (Dr. R. E. Hungate)

National Institute of Dental Research, Laboratory of Microbiology (Dr. Morrison Rogosa)

Laboratory of Parasitic Diseases, NIAID (Dr. Charles S. Richards)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	18/12	-	-	18/12
Professional	18/12	-	-	18/12
Other	-	-	-	-

Project Description:

Objectives:

To examine the ultrastructure of microorganisms, in self-generated and collaborative studies, with particular emphasis on bacterial cell walls and membranes, their related antigens, and aspects of their participation in growth, division, genetic interchange, bacteriophage absorption, and the like.

Methods Employed:

Standard or newly-developed methods of bacteriology, serology, phage virology, microbial genetics, biochemistry, and electron microscopy. When appropriate, special methods of antibody labelling, cytochemistry, or other techniques of marking for electron microscopy.

Major Findings:

(a) Bacillus subtilis mutants. Last year's studies of ultrastructural changes in a temperature-sensitive mutant were completed and published. Identical ultrastructural changes were seen in another strain to which the mutant locus was transferred. Examination of salt-requiring mutants (from Dr. H. J. Rogers, NIMR, Mill Hill, London) affirmed a similar rod-to-sphere conversion and abnormal cross-walls in the restrictive condition (no salt), but the losses of cell-wall layers and periplasm, and generalized wall thickening found in the temperature-sensitive mutant, were not seen. Instead, double-thicknesses of walls with frequent splitting, were common; and peculiar intra-membranous lesions of a periodic and presumably lipid nature, were seen in mesosomes. Currently, a filamentous (sep^-) temperature-sensitive mutant (from Dr. N. Mendelson, U. of Arizona) is being studied. Preliminary results verify the non-separate and multi-nucleated nature of the excessively long filaments produced at 45°C (but not at 30°C). Cross-walls are not visible by 60 minutes after the shift to 45°C, some lysis occurs, filaments grow in length; and on return to 30°C, new cross-walls in surviving filaments are seen after 30 minutes and are completed by 60 minutes. The times of cross-wall disappearance, and reappearance and completion, correspond to the times of a sharp increase or decrease, respectively, in the plotted modal cell lengths. No marked alterations of cell wall ultrastructure, except for some generalized thickening, irregularity, or occasional layering at 45°C, is apparent. Supernumerary cross-walls and prominent abnormal mesosomes are occasionally seen on return to 30°C.

(b) Staphylococcus aureus mutants. A series of salt-dependent or methicillin-resistant staphylococci (from Dr. S. Seligman, Downstate Medical Center, N.Y.) were examined in sections from samples grown for varying times at different temperatures, and with or without salt. Preliminary results show that there are strain-dependent differences in cell-wall thickness and ultrastructure. The influence of salt varies with strain--its presence enhancing the appearance of spherical clusters of non-separating cells with random cross-walls in some strains, and its absence producing similar tendencies, cell wall layering, or bizarre cytoplasmic effects, in others. Continued study and exhaustive analysis are needed before meaningful conclusions can be made.

(c) Escherichia coli mutants. The wild-type (wt) strain of E. coli B and three T-phage-resistant mutants (from Dr. J. G. Michael, U. of Cincinnati) were grown, fixed in osmium, and examined in sections. The wt showed normal gram-negative cell architecture, but in this and the others, no distinct mucopeptide layer in the periplasm (as seen in some other E. coli) was visible. Mutant #21 (T2-resistant) was also of normal structure. Mutant #261 (T2- and T6-resistant) showed a moderate amount of protoplast plasmolysis within the intact cell wall. However, in mutant #61 (T6-resistant), there was extensive plasmolysis--often at the poles, and resulting in a truncated appearance. Large spherical spheroplasts, often losing the wall layer and giving rise to protoplasts and empty membrane spherules, were common. Rarely, peripheral pockets of plasmolysis contained what appeared to be liberated mesosome vesicles, and mesosome-like membrane configurations were often seen at cross-wall sites in intact cells. No differences in wall ultrastructure were seen among the 4 strains, and it would not be expected that minor chemical changes involved in altering phage receptors could be resolved by electron microscopy.

(d) Reaggregated membranes. In collaboration with Dr. Razin and Dr. Chanock (LID) and Dr. Prescott, we examined the reaggregation of membrane proteins and lipids from different sources. Detergent solubilization of mycoplasma membranes (from Mycoplasma pneumoniae and Acholeplasma laidlawii) resulted in protein and lipid fractions. The latter were further purified to glycolipids. Ultrathin sections of A. laidlawii protein showed amorphous clumps. Sections of M. pneumoniae glycolipid showed globules with concentric lamellar spacing of 37 A°. Following mixture of these two components and dialysis (to remove detergent) against a Mg^{2+}-containing buffer, thin sections showed typical "triple-layered" membrane profiles with a center-to-center spacing of 54 A°. The appearance and dimensions of the "hybrid" membranes closely resembled those of the original or reaggregated homologous membranes. It is believed that the reaggregated physical structures account for increased antigenicity of the glycolipid, as reported elsewhere (see Project No. NIAID-64C).

(e) Membranes and mesosomes of Staphylococcus aureus ATCC 6538P. Progressive stages, from the initiation of protoplasting through the final separation and purification of membranes and mesosomal vesicles, were followed by electron microscopy of ultrathin sections. As the cell wall is attacked by a muralytic enzyme, eversion of mesosomal sacs extrudes the numerous mesosomal vesicles (of 35-50 nm diameter) into peripheral submural pockets and, as wall is ruptured, to the medium. At this stage, which is achieved nearly completely in 60 minutes, the mixture contains intact protoplasts, cell wall fragments, mesosomal vesicles, some larger vesicles, and occasional intact cells. By differential and density-gradient methods already described (see Project No. NIAID-63B, 1969-70), following nuclease digestions, homogeneous preparations of mesosomal vesicles and membranes were obtained. The former are membrane-bounded, of the same size as in intact cells, and have a homogeneous content which distinguishes them from larger vesicles occasionally pinched off from protoplasts. If magnesium was omitted in the preparation, no mesosomal vesicles were recovered. However, its omission freed membranes of otherwise adherent ribosomes. The membrane preparation, which resulted from controlled protoplast lysis, consisted of only typical triple-layered membrane profiles. Enzymatic and chemical analyses of the membrane and mesosome vesicle fractions were previously initiated (Project No. NIAID-63B, 1969-70) and are continuing (Project No. NIAID-63B, 1970-71).

(f) Electron microscopic studies in support of other projects.

1. Enzymatic lysis, and recovery of protoplasts and mesosomal vesicles of Group A streptococci, were followed. Optimal conditions were found to be 30% raffinose, 0.1 M magnesium, and pH 6-7.(Project No. NIAID-63C, Schade)

2. Inter-group-infecting bacteriophages from streptococcal Groups A and G were compared at high resolution by negative staining. They are ultrastructurally, as well as serologically, related. (Project No. NIAID-65A, Colon and Leonard)

3. Examination during reduction of optical density of genetically competent (but not incompetent) cultures of Group H streptococci revealed lysis restricted to the cross-walls of the cells. Similar lysis of non-competent cells was produced by supernates of competent lysates. (Project No. NIAID-65C, Ranhand)

4. When Histoplasma capsulatum yeast cells were grown in the presence of partially inhibitory concentrations of saramycetin, electron microscopy revealed that the proportion of apparently damaged cells was a function of the concentration of the antibiotic. Evidence of early damage consisted of irregularity of outline, followed by progressive lysis of internal structures leading to residual dense granules, membranes, and the cell wall. Normal yeast cells of the strain studied were found to possess intranuclear bodies of a type apparently not previously described. (Project No. NIAID-78A, Lones)

5. Cultures of anaerobic rumen bacterial (Ruminococcus albus, Peptostreptococcus elsdenii) were received from Dr. Hungate (U. of California, Davis), Dr. Bryant (U. of Illinois), and Dr. Rogosa (NIDR), and examined in sections. Ruminococci possess a gram-positive type of cell wall structure, and one strain is heavily capsulated. P. elsdenii has the characteristic gram-negative architecture: this, and biochemical and physiologic characteristics, have led to its redescription by Dr. Rogosa as a new genus, Megasphaerus, gen. nov. These anaerobes had not been previously examined by electron microscopy.

6. Sections of sub-epidermal nodules from an African snail (Bulinis by Dr. Richards (LPD, NIAID) showed presumptive bizarre bacteria in loculated "lesions." Electron microscopy of new sections, negatively-stained and metal-shadowed dissected preparations, and gram-staining and phase microscopy, demonstrated a gram-negative non-motile bacillus with a "head" end from which

protrude 25-30 long and relatively rigid and wide (325 A°) "filaments." Within the tissue, apparent stages of development lacking "filaments" and "head" differentiation were seen. The "filaments" are composed of several smaller filaments wound counter-clockwise around a central core. Preliminary attempts at cultivation were unsuccessful. This bacterium meets no known morphologic descriptions and its taxonomic relationships are uncertain. Studies on cultivation and infectivity, as well as continued ultrastructural investigation, are in progress.

7. Attempted cytochemical localization in Staphylococcus aureus of iron, and of redox activities by tetranitro blue tetrazolium and tellurite reduction, were equivocal. Tellurium was best, and tests will be repeated with other staphylococcal strains and under different conditions. Use of the cationic dye, Alcian Blue, for possible cytochemical study of bacterial cell walls, produced results that are currently uninterpretable.

8. For reasons of monitoring procedures or for basic information, sections were made and examined of Vibrio cholerae, an asporogenous "lysogenic" strain of Bacillus cereus, and streptococcal L forms (Strep. MG). Preparation and purification of L form membranes was monitored by examining negatively-stained preparations.

Significance to Bio-Medical Research and the Program of the Institute:

The utility of electron microscopy as a monitoring or supportive approach is clear from the natures and results of the studies in which we have collaborated (See the appropriate Project Reports herein). Our direct approach to the correlation of structure and function continues to be examination of available bacterial mutants in comparison with normal strains. In extension of prior studies of mutants showing conditional changes in shape, cell wall structure, and division, we are now studying other mutants of the same bacterium (Bacillus subtilis) in which the conditionally-expressed changes relating to cell division, and cell walls, are quite different. We anticipate that the study of abnormalities, arising through mutations or other means, will continue to be most useful in defining normal processes of bacterial growth and division.

Proposed Course of Project:

Studies of normal and mutant bacteria, and of isolated bacterial components, to be continued.

Honors and Awards:

Continuing memberships:

Library Advisory Committee, NIH

Vaccine Development Committee, NIAID, NIH (terminated March 1971)

Project Officer, NIAID Research Contract PH43-68-83,
University of Chicago

Honors and Awards Cont.

Presented paper on "Ultrastructure of a Temperature-sensitive Rod$^-$ Mutant of Bacillus subtilis," at the Xth International Congress for Microbiology, Mexico City, Aug. 9-15, 1970.

Presented the Annual Lecture on "The Importance of the Bacterial Cell Wall" at the South Dakota Chapter of Sigma Xi, Vermillion, South Dakota, November, 1970.

Invited discussant at the Symposium on Streptococcal Disease, University of Minnesota, Minneapolis, Minn., May 12-14, 1971.

Invited participant, Round Table on "Cell Division in Bacteria," Annual Meeting, ASM, May 6, 1971.

Presented seminar on "Cell Wall Ultrastructure and Mutations," Dept. of Microbiology, University of South Dakota, Medical School, Vermillion, S.D., November, 1970.

Publications:

Cole, R. M., Popkin, T. J., Boylan, R. J., and Mendelson, N. H.: Ultrastructure of a temperature-sensitive rod$^-$ mutant of Bacillus subtilis. J. Bacteriol. 103: 793-810, 1970.

Ranhand, J. M., Theodore, T. S., and Cole, R. M.: Protein difference between competent and noncompetent cultures of a Group H streptococcus. J. Bacteriol. 104: 360-362, 1970.

Cole, R. M., Popkin, T. J., Prescott, B., Chanock, R. M., and Razin, S.: Electron microscopy of solubilized Acholeplasma laidlawii membrane proteins reaggregated with Mycoplasma pneumoniae glycolipids. Biochim. Biophys. Acta 233: 76-83, 1971.

Ranhand, J. M., Leonard, C. G., and Cole, R. M.: A comparison of streptococcal competence factors produced by strain Challis in chemically defined and complex media. J. Gen. Microbiol. (in press) 1971.

Ranhand, J. M., and Cole, R. M.: Demonstration of transforming DNA in an autolysate of a Group H streptococcus. J. Bacteriol. (in press) 1971.

Ranhand, J. M., Leonard, C. G., and Cole, R. M.: Autolytic activity associated with competent Group H streptococci. J. Bacteriol. 106: 257-268, 1971.

Leonard, C. G., Ranhand, J. M., and Cole, R. M.: Competence factor production in chemically defined media by noncompetent cells of Group H streptococcus strain Challis. J. Bacteriol. 104: 674-683, 1971.

Publications cont.

Theodore, T. S., Tully, J. G., and Cole, R. M.: Polyacrylamide gel identification of bacterial L forms and *Mycoplasma* species of human origin. *Appl. Microbiol*. 22: 272-277, 1971.

Theodore, T. S., Popkin, T. J., and Cole, R. M.: The separation and isolation of plasma membranes and mesosomal vesicles of *Staphylococcus aureus*. *Preparative Biochem*. (in press) 1971.

Colón, Alba E., Leonard, C. G., and Cole, R. M.: Lysis and lysogenization of Groups A, C, and G streptococci by a transducing bacteriophage induced from a Group G streptococcus. *J. Virology* (in press)

Serial No. NIAID 65A
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Genetics of Group A Streptococci

Previous Serial Number: Same

Principal Investigator: Alba E. Colón

Other Investigators: Carmen G. Leonard
Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	18/12	-	-	18/12
Professional	15/12	-	-	15/12
Other	3/12	-	-	3/12

Project Description:

Objectives:

To continue studies on the development of transduction systems within different streptococcal groups and on intergroup transduction. The work will include studies on phage specificity and the nature of phage receptor sites. Intergroup transduction may permit the exchange between groups of virulence factors such as M proteins, hemolysins, and other bacterial antigens.

Methods Employed:

The usual methods in microbial genetics, bacteriophage isolation and identification were employed.

Major Findings:

Last year we reported the isolation of a temperate bacteriophage (GT-234) from a Group G streptococcus following ultraviolet irradiation. Further work was done with this and other phages. After several single passages in a Group G indicator strain, this phage formed plaques in 3/14 Group A strains, 3/15 Group C strains, and 2/11 Group G strains - but not in streptococci of other serogroups. Following propagation in each of the sensitive strains, the progeny from each was shown to be the same phage by 1) adsorption and plaque formation in each of the other groups; 2) lysogenization in each of the other groups; 3) high titers of infection of each serogroup, regardless of the group of the propagating strain; and 4) neutralization of infection in each of the other groups by antisera raised against the phage propagated in Group G. Other studies showed that Group A virulent phage (A 25), as well as Group A temperate phage (AT-298), could also infect strains of Group C, but not of Group G. Like A 25 and AT-298, GT-234 is a transducing phage capable of transducing the streptomycin resistance marker within Group A strains of streptococci. Since these transducing bacteriophages can infect streptococci of Groups A, C and G, it seems feasible that they can also transduce among these groups. Work along these lines is now in progress.

Significance to Bio-Medical Research and the Program of the Institute:

As streptococcal phages have been considered group specific, these results indicate a need for reassessment of group specificity and phage receptors among streptococci of Group A, C and G. They also present possibilities for study of genetic exchange among these groups. We hope that future studies will lead to better understanding of the sero-identification and pathogenicity of Group A streptococci.

Proposed Course of Project:

Studies in intergroup transduction between Groups A, C and G are in progress using streptomycin resistance as the marker. We plan to use other antibiotic markers, such as tetracycline and erythromcin. The successful development of intergroup transduction would allow the use of different bacterial antigens, such as M-protein, wall carbohydrates, etc. as markers for transduction.

Honors and Awards:

Miss Colon was invited to join the honorary scientific society for professional women, Sigma Delta Epsilon.

Publications:

Colon, Alba E., Leonard, C. G., and Cole, R. M.: Lysis and lysogenization of Groups A, C, and G streptococci by a transducing bacteriophage induced from a Group G streptococcus. J. Virology (in press).

Serial No. NIAID-65B
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Identification of Bacterial L Forms and _Mycoplasma_ Species.

Previous Serial Number: Same

Principal Investigator: Theodore S. Theodore and Joseph G. Tully

Other Investigators: Roger M. Cole

Cooperating Units: Charles Zierdt
Clinical Center
Clinical Pathology Department

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	6/12	-	-	6/12
Professional	3/12	-	-	3/12
Other	3/12	-	-	3/12

Project Description:

Objectives:

To develop a method which is applicable to both clinical and basic research studies for the identification of bacterial L forms and _Mycoplasma_ species.

Methods Employed:

Routine bacterial and cultural procedures, polyacrylamide gel electrophoresis and analysis by densitometry.

Major Findings:

A. _Identification of L forms and Mycoplasma species_. In the past year we have continued our studies on the identification of L forms and _Mycoplasma_ species using gel electrophoresis. The electrophoretic patterns of _Staphylococcus aureus_ Smith parent (ATCC 19636) and L form (ATCC 19640); and several mycoplasmas (M. lipophilium MaBy, 3 Navel strains, and 6 sheep and goat strains) were analyzed and added to our list of reference strains. Also, we routinely monitored the purity of L form cultures used in other studies (Project No. NIAID-65I, Dr. Birnbaum).

B. Characterization of cell wall defective microorganisms (transitional forms). In addition to the differentiation of L forms and Mycoplasma species, we applied our method to the identification of cell wall defective microorganisms which reportedly had been isolated from patients with subacute bacterial endocarditis. Initially, these microorganisms were identified as transitional forms of Corynebacterium acnes, an anaerobic diphtheroid. (Dr. Zierdt, CC,CPD)

Gel electrophoresis of these transitional forms showed that (1) they could be classified into 4 distinct groups, (2) none resembled the suspected parent C. acnes, and (3) they were not related to any of our bacterial or L form reference strains. As yet, the parental origin of any of these transitional forms is not known (note of added proof: Dr. M. Mandel, Houston, Texas, using guanosine-cytosine base rations as a measure of genetic relatedness has verified our findings that the transitional forms are not related to C. acnes).

Significance to Bio-Medical Research and the Program of the Institute:

The identification of L forms and Mycoplasma, and the differentiation between them, is very difficult because of their biological similarities. The aim of our investigations was to develop a method which was rapid and simple for the identification of these and other microorganisms when isolated from some chronic and acute diseases. Such a method is polyacrylamide gel electrophoresis. It excludes the necessity for expensive and lengthy procedures such as preparing antisera or radioactively labelled DNA for hybridization studies and its utility has been shown.

Proposed Course of Project:

As cultures of L forms and Mycoplasma become available, we will establish a reference index of standard patterns for identification.

Honors and Awards:

Consultant, National Cancer Institute, Viral Biology Branch. Special Animal Leukemia Ecology Segment. December 1970. University of California, Davis, California. The use of polyacrylamide gel electrophoresis for separating and identifying membrane proteins of several feline and sub-human primate oncogenic viruses. (Theodore)

Publications:

Theodore, T. S., Tully, J. G., and Cole, R. M.: Polyacrylamide gel identification of bacterial L forms and Mycoplasma species of human origin. Appl. Microbiol. 22, 272-277, 1971.

Serial No. NIAID-65C
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Competence Development and Genetic Transformation in Group H Streptococci

Previous Serial Number: Same

Principal Investigator: Carmen G. Leonard and Jon M. Ranhand

Other Investigators: Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	54/12	-	-	54/12
Professional	22/12	-	-	22/12
Other	32/12	-	-	32/12

Project Description:

Objectives:

To define in biochemical, physiological, and morphological terms the nature of the competent state as it exists in the Group H streptococci and to define the chemical nature of competence inducing factors. Competence is the ability of bacterial cells to react with exogenously supplied deoxyribonucleic acid (DNA) and become genetically transformed.

The nature of competence is not fully understood in any of the known transformable systems. The Group H streptococci are an ideal system to study the mechanisms involved that lead to the competent state. These steps include: (a) competence factor production, (b) cell binding of factor and the nature of the essential receptors, (c) the nature of induction by competence factor of the subsequent steps in competence development which require protein synthesis, (d) DNA uptake by competent cells, and (e) DNA integration and expression of the newly acquired traits. Work is in progress in each of the above steps.

Methods Employed:

Standard bacterial and chemical methods are used. We developed procedures for the isolation and purification of competence factors by modifications of known techniques of column and thin layer chromatography, electrophoresis, and isoelectric focusing.

Major Findings:

Part A (Leonard)

We reported last year the development of defined media for growth, competence factor production, and competence development by strain Challis. The availability of these defined media permitted studies that showed that competence factor production is independent of competence development. Glutamate, which is essential for transformation, is not required for the elaboration of competence factors. These findings now permit independent studies of the various stages and mechanisms which terminate in genetic transformation in Group H streptococci.

This year, we developed standard procedures for the isolation and purification of competence factors obtained from culture supernatant fluids of strain Challis grown in our defined medium. A major improvement in the purification procedure described in last years report was the finding that extraction of the competence factor preparation at 60°C, pH 2.0 for 20 min. solubilized the factor, while the bulk of the impurities remained in the pellet fraction. This pH 2.0 product is dialyzable, has a molecular weight of about 5,000, and behaves like a peptide since its biological activity is destroyed by proteolytic enzymes. Such competence factor preparations appear homogeneous by both polyacrylamide gel and paper electrophoresis. Isoelectric focusing of competence factor prepared in this manner shows it to contain at least 6 different bands, one of which contains most of the biological activity. This band has been isolated and it is now being chemically analyzed.

Part B (Ranhand)

We observed in the past year a number of new and interesting phenomena associated with the bacterial competent state as it exists in the Group H streptococci strains Wicky and Challis. First was the demonstration by polyacrylamide-gel electrophoresis of the appearance of a new protein that is present only in competent cells. In streptococcus Wicky, which cannot develop competence spontaneously, the new protein was made in response to competence inducing factor(s) derived from strain Challis. The induction of competence, as well as of the new protein, was inhibited by chloramphenicol. Furthermore, it was shown that any treatment that prevents the Wicky cells from becoming competent also prevents the elaboration of the new protein. Challis cells which are spontaneously competent had, as expected, the new protein.

Secondly, a lytic phenomenon also specific for competent cells was discovered. This important observation allows us to hypothesize a mechanism on how the DNA molecules pass through the bacterial cell wall. Localized autolysis of the cell wall might expose the underlying membrane to the DNA and thereby enable it to be transported into the cell by yet unknown mechanisms. Autolysis of competent cells is only observed at basic pH values (7.5-9.1) and is enhanced by reducing agents such as 2-mercaptoethanol. Electron microscopic observation of autolysed cells showed that the cross-wall region is the target for the enzyme. Furthermore, the action of the enzyme in competent cells is not complete in that cell wall hemispheres remain even after prolonged incubation. This limited action is the type that one would expect if local autolysis is involved in competence. Ultra thin sections of lysed cells revealed that the cell wall is nicked to such an extent that leakage of the cell's cytoplasm and DNA occurs.

Thirdly, it was found that the supernatant fluids of lysed cells contain transforming DNA of very high quality. This last observation is a very important one because it shows that DNA can pass through the autolysed cell wall. Moreover, this DNA is of very high molecular weight. The relatively easy method by which the cells' DNA can be recovered lends itself to studies of the DNA per se. Work is in progress on the characterization of the streptococcal DNA both by genetic analysis as well as physical analysis.

Significance to Bio-Medical Research and the Program of the Institute:

Soluble competence factors (CF), although reported in several transformable bacteria, have not been completely purified; therefore, their chemical natures are uncertain and their modes of action cannot be adequately studied. Our use of defined media, establishment of optimum conditions for CF production, determination of CF production as a step separable from subsequent stages of competence, and improvements in large-scale isolation and purification procedures, bring us closer to final chemical and physical characterization of streptococcal competence factor. The discovery of a new protein and autolysis restricted to competent cells, provide additional and clearer evidence of competence-related activities suggested to exist in some other bacterial transforming systems. Both these phenomena appear as a result of CF binding to cells and both are apparently essential to the ultimate steps (DNA uptake and integration) that define competence. They, therefore, supply new criteria useful in determining the site and mode of action of CF, and in defining and separating the number and nature of the events that determine the state of genetic competence in bacteria.

Proposed Course of Project:

We plan to continue our work on the chemical nature of competence factor(s) and to study the initial steps involved in its uptake and utilization by non-transformable Wicky cells. In this connection, we plan to investigate in detail the nature and location of the newly synthesized protein. We also want to establish the role played by the autolysin in competence development. The DNA isolated from the non-transformable strain Wicky is also being characterized.

Honors and Awards:

Invitation to join the National Honorary Society for Graduate Women, Sigma Delta Epsilon. (Leonard)

Presented paper, "Studies on Competence Factors Produced by Group H Streptococci in Chemically-Defined Medium," at the Xth International Congress of Microbiology, Mexico City, Aug. 9-15, 1970. (Leonard)

Lecturer - Foundation for Advanced Education in the Sciences. (Ranhand)

Presented seminar on "Bacterial Transformation in Group H Streptococci," at Johns Hopkins University School of Hygiene, May 10, 1971. (Ranhand)

Publications:

Leonard, C. G., Ranhand, J. M., and Cole, R. M.: Competence factor production in chemically defined media by noncompetent cells of Group H streptococcus strain Challis. J. Bacteriol. 104: 674-683, 1970.

Ranhand, J. M., Theodore, T. S., and Cole, R. M.: Protein difference between competent and noncompetent cultures of a Group H streptococcus. J. Bacteriol. 104: 360-362, 1970.

Ranhand, J. M., Leonard, C. G., and Cole, R. M.: Autolytic activity associated with competent Group H streptococci. J. Bacteriol. 106: 257-268, 1971.

Ranhand, J. M., and Cole, R. M.: Demonstration of transforming DNA in an autolysate of a Group H streptococcus. J. Bacteriol. 106; May (in press)

Ranhand, J. M., Leonard, C. G., and Cole, R. M.: A comparison of streptococcal competence factors produced by strain Challis in chemically defined and complex media. J. Gen. Microbiol. (in press)

Colon, Alba E., Leonard, C. G., and Cole, R. M.: Lysis and lysogenization of Groups A, C, and G streptococci by a transducing bacteriophage induced from a Group G streptococcus. J. Virology (in press)

Serial No. NIAID-65D
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Reversion of Group A Streptococci

Previous Serial Number: Same

Principal Investigator: Dr. Harry A. Gallis

Other Investigator: Dr. Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	0/12	-	-	0/12
Professional:	0/12	-	-	0/12
Other:	0/12	-	-	0/12

Project Description:

See 1970 Annual Report.

Project terminated.

Serial No. NIAID-65F
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The cell wall constituents found in a stabilized L form.

Previous Serial Number: Same

Principal Investigator: James R. King and Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned To Project	Guest Workers	TOTAL
Total	6/12	-	-	6/12
Professional	6/12	-	-	6/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the presence of, and to characterize any cell wall constituents in a stable L form of a Group D streptococcus (Strain F24).

Methods Employed:

All purification and analytical procedures are modifications of previously existing methods. Complex and lengthy methods of column chromatography, paper chromatography, colorimetry, chemical extraction, and immunochemistry were used. Only a general version of methods is given in this report.

Polysaccharides were extracted from washed parent strain organisms or L forms by alkali treatment. Contaminating protein and other materials were removed from the alkali extracts by selective precipitations and dialyses of the crude polysaccharide preparations with, (1) 95% ethanol:ether (3/1) to recover all polysaccharide material and macromolecular contaminants, (2) an equal volume of 15% trichloric acid (TCA) added to the dissolved ethanol:ether precipitated material to remove proteins and nucleic acids, (3) dialysis of the neutralized purified polysaccharide with water to remove TCA, and (4) lyophilization of the purified polysaccharide. Such preparations were analyzed for sugars and amino sugars by standard procedures, especially for rhamnose whose presence in parent material and absence in L form material was reconfirmed.

For immunochemical analysis, the purified polysaccharides were dissolved in phosphate-buffered saline and tested for immunochemical reactions against rabbit antiserum directed against strain F24 cell walls. Three standard immunochemical procedures were used to analyze the polysaccharides: (1) precipitin reactions in Ouchterlony plates to compare identities of the polysaccharides from parent and L form, (2) quantitative precipitin reactions to determine the amount of unknown L form polysaccharides which would cross react with parent cell wall polysaccharide, and (3) inhibition tests of the quantitative precipitin reaction to detect any non-precipitable polysaccharide antigen which would cross-react with the parent polysaccharide antigen.

Major Findings:

A thorough serological comparison of L form and parent strain polysaccharides was performed. Several parent strain polysaccharides were identified serologically in Ouchterlony plates. When similarly prepared L form polysaccharide was tested, precipitates were visible. Three different conclusions are possible from the results of these experiments: (a) there was antigen excess, which caused the specific precipitates to dissolve, (b) there was no serologically reactive material present in L form polysaccharide, or (c) there was antigen present which combined with the antibody but caused no precipitate. The problem was solved by the inhibition test mentioned above under Methods. Inhibition tests with L form polysaccharide antigen shared no inhibition of the parent polysaccharide-antibody precipitin reactions. These results show the absence of serologically reactive polysaccharide in the L form preparations.

These serological results, together with previous analytical work (seen in last year's annual report) suggest that the L form has a total disorganization of its cell wall polysaccharide synthetic mechanism, because (1) demonstrable cell wall polysaccharide was missing from the L form and (2) the L form polysaccharides were chemically and immunochemically distinct from the parental polysaccharides.

Significance to Bio-Medical Research and the Program of the Institute:

Many investigators feel that L forms play a significant role in some chronic recurrent infectious diseases, because the L forms revert from their normally non-detectable non-virulent form to the parent bacterial form which is virulent. Stable L forms are generally non-pathogenic--most probably because only bacterial forms can survive in large numbers due to the protective effect of their cell wall. This study attempts to define the chemical nature and quantity of any cell wall constituents which may be present in a stable

L form, so that eventually correlations may be drawn between cell wall constituents and pathogenicity or virulence of the L forms. Another goal of these studies is to determine the L form's lesion in biosynthesis of cell wall, so that the reversion process may be understood *in vitro* first, and subsequently *in vivo*. If the chemical reactions in the reversion process are elucidated, then the role of bacterial L forms in infectious diseases may be more clearly defined through controlled experiments.

Proposed Course of Project:

At present, most of the cell wall polymers of the parent strain F24 and its derived L form have been well characterized, chemically or immunochemically. Other major polymeric substances, such as teichoic acid, remain to be examined. Isolation of these polymers is under way, and special attention has been given to lipid complexes-- characterized by the presence of C55 isoprenoid material. The presence of these compounds may be directly related to the ability of bacterial cells to synthesize cell wall, based on current knowledge in the field of cell wall biochemistry.

Honors and Awards:

Presented paper on "Differences in the carbohydrate content of *Streptococcus faecium* F24 and its stable L form," at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., May 2-7, 1971. (King)

Publications:

None

Serial No. NIAID-65G
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Characterization of biophysical and physiological properties of L forms.

Previous Serial Number: Same

Principal Investigator: James R. King

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	10/12	-	-	10/12
Professional	10/12	-	-	10/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the comparative genetic map of a Group D streptococcus (F24) and its stable L form--particularly in those genetic loci controlling cell wall synthesis and cell division; to determine the role of any membrane-located binding sites for DNA which function in control of orderly cell division by bacteria; to study the biophysical properties of L forms of bacteria; and to study the physiological response of L forms to in vivo environments.

Methods Employed:

Routine bacteriological culture techniques; enumeration of viable organisms in cultures; estimation of bacterial cell mass based on physical properties of the organisms, e.g., turbidity measurements compared to dry weight of a culture; colorimetric determination of protein content of bacterial cell mass; chemical extractions of cells; ultracentrifugation;

monitoring purity and concentrations of DNA by ultraviolet spectrophotometry; phase microscopy; sucrose density gradient centrifugation; fractionation of bacterial cells; use of specific metabolic inhibitors to elucidate the relationship between bacterial structure and function; turbidimetric analysis of bacterial population growth; microscopy (direct light); controlled use of antibiotics to determine metabolic pathways.

Major Findings:

(A) Experiments were performed to corroborate work done with Streptococcus faecium F24 and to conclusively show why synchronization of chromosomal replication (a prerequisite for mapping a bacterial genome by sequential mutation) could not be obtained with para-fluorophenylalanine (FPA). Escherichia coli (known to be inhibited by FPA in synthetic medium and subsequently synchronized after release from the inhibitor) was virtually refractile to the action of 0.02-0.08 M FPA in complex medium, while the organism was at least 50% inhibited by 0.004-0.016 M FPA in synthetic medium. Growth kinetics of E. coli during and after inhibition by FPA, together with previous results from the enterococcal system, show that synchronization of chromosomal growth--a result of DNA inhibition--is unattainable when complex medium is used for treatment of these organisms.

(B) An improved light microscopic technique was developed for simultaneous observation of large numbers of L forms grown in broth. A typical broth suitable for the propagation of L forms contains a high concentration of salt, animal tissue extracts, and macromolecules--all of which lead to artifacts in light microscopy when a sample from a broth culture is applied to a glass slide and fixed to the surface. I have modified standard electron microscopic techniques (as used by D. G. Sharp, G. J. Galasso, and K. O. Smith) for rapid mounting, removal of inert material, and staining of preparations. Using my technique, an investigator can examine over 100 L forms simultaneously in focus instead of in constant Brownian motion. The technique is currently being used to examine many L form bodies in the same small sample during growth after apparent synchronization by FPA.

(C) A physicochemical association of DNA and membrane has been documented in some bacteria and is considered necessary in some theories that explain segregation of two daughter genomes. Further attempts with Streptococcus faecium F24 and its L form fail to show reproducible definitive membrane-DNA complexes. Therefore, no conclusions can be drawn concerning comparative DNA distribution in dividing enterococcal parent strain and L form. These organisms grow and divide (a prior evidence for DNA replication and distribution), but the involvement of the membrane in the mechanism of distribution is still unknown.

(D) During the studies presented above, biophysical data were needed to confirm or deny an hypothesis that simple centrifugation will kill a specific strain of L forms. Other workers have shown conclusively that centrifugation significantly reduces the viable numbers of Group A streptococcal L phase variants in a suspension. However, the results obtained in this Laboratory with an enterococcal L form are diametrically opposed to this work which has been long regarded as almost axiomatic for all streptococcal L forms. The enterococcal L form, strain F24, showed no loss of viability upon repeated centrifugation and subsequent resuspension of the organisms in growth medium.

(E) Another generally recognized property of streptococcal L forms is dependence on an osmotic stabilizer in the medium. It has been assumed that the added substance prevents lysis of osmotically fragile bodies under any conditions. However, experiments in this Laboratory suggest that a given "osmotically protective agent" has none of the desired effect in a different medium. For example, ammonium chloride is effective in preserving the viability of enterococcal L forms in complex broth, but there is no detectable protective effect when the organisms are transferred to a partially synthetic medium containing the same salt. Cell death (greater than 90% reduction in viability) is not relatable to potential growth inhibitors in the partially synthetic medium because the cell mass decreases quickly--a condition which indicates lysis.

Significance to Bio-Medical Research and the Program of the Institute:

Throughout the history of L form research, there has been a tendency among scientists to (1) classify these organisms together, ignoring the differences in biophysical properties where those properties were known and (2) extrapolate from one organism to another organism when those biophysical properties were not known. Clinical researchers have been no different. Frequently, inspection of the methods in a typical clinically-oriented publication reveals the use of only one type of osmotically protective agent in an isolation medium. These studies indicate that better recovery of different L forms from infectious diseases might be accomplished if more than one isolation medium were used. A more thorough biophysical study of several L forms should result in elucidation of those biophysical parameters affecting the viability of L forms.

Proposed Course of Project:

Genetic mapping of the parent and L form organisms appears to be impossible with existing genetic techniques. The DNA-membrane complex (relatable to orderly cell division) appears to be missing in a recoverable state in both parent and L form. Therefore, the original goals are not attainable with present methods. However, a thorough biophysical and physiological characterization of L forms will be attempted. Special attention will be directed toward determining those parameters affecting recovery of L forms as viable units from various animal body fluids. The initial work in the project was performed with enterococcal L forms, but other organisms will be examined.

Honors and Awards:

None

Publications:

None

Serial No. NIAID 65H
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical Evidence for the Degradation of Streptococcal Cell Walls by Human Polymorphonuclear Leukocytes.

Previous Serial Number: Same

Principal Investigator: Dr. Richard A. Getnick

Other Investigators: Dr. Alan D. Glick
Dr. Roger M. Cole

Cooperating Units: Laboratory of Biochemistry and Metabolism, NIAMD
(Dr. Gilbert G. Ashwell)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	11/12	-	-	11/12
Professional	11/12	-	-	11/12
Other	-	-	-	-

Project Description:

Objectives:

To devise a biochemical method by which the degradation of Group A streptococcal cell walls by human polymorphonuclear leukocytes (PMN's) can be followed.

Methods Employed:

The Group A streptococcal cell wall has a complex protein component and polysaccharide fraction. The polysaccharide fraction is separable into two components: group-specific carbohydrate and mucopeptide. Rhamnose is the major constituent of the C-polysaccharide (Krause, Bacteriol. Rev. 27: 369-380, 1963) and is not present in any other part of the streptococcal cell. Therefore, demonstrating that rhamnose is released during streptococcal degradation by human PMN's would provide chemical evidence for cell wall destruction.

Group A streptococci in the stationary phase were incubated at 37°C with human white blood cells (WBC's) obtained by dextran sedimentation of heparinized whole blood (10 colony forming units of bacteria per WBC). In the presence of Hank's BSS (without phenol red) to which human plasma to give a final concentration of 20% had been added (Hank's-plasma), uptake was excellent. After a 5 hour incubation in Hank's-plasma the suspension was centrifuged to bring down all WBC's (most of which contained intracellular streptococci) and extracellular streptococci. The supernatant containing any released rhamnose was removed and stored in a freezer. The pellet was resuspended in distilled water and the suspension freeze-thawed to lyse the WBC's and release any intracellular rhamnose. Streptococci and WBC membranes were removed by centrifugation and this supernatant was combined with the first supernatant.

Originally the Dische and Shettles method for methylpentoses was used to detect any rhamnose present in the combined supernatant (J. Biol. Chem. 175: 595-603, 1948). The major drawback with this method was its lack of specificity. Fucose (from red blood cell membranes) contaminated the experimental supernatant and could not be distinguised from rhamnose.

Another approach was attempted using a crude preparation of the enzyme rhamnose isomerase to convert rhamnose to rhamulose, a keto sugar (Wilson et al, J. Bact. 73: 410-414, 1957; Green et al, J. Biol. Chem. 219: 557-568, 1956). The cysteine-carbazole reaction to measure rhamulose (Dische et al, J. Biol. Chem. 192: 583-585, 1951) was then used.

However, the combined supernatant contained several major constituents (salts; glucose; and hemoglobin and plasma proteins) which had to be removed before the expected microgram quantities of rhamnose could be detected by the enzymatic and colorimetric method described above. Glucose was converted to gluconic acid by glucose oxidase. Trichloroacetic acid was used to precipitate glucose oxidase after the reaction was complete and to precipitate hemoglobin and plasma proteins. Gluconic acid and other ions contained in Hank's-plasma were removed by passage through ion exchange resins.

The specimen was then hydrolyzed with .5N H_2SO_4 to convert any rhamnose present in the polymeric form to the monomer (the enzyme rhamnose isomerase converts only monomeric rhamnose to rhamulose). To remove the sulfuric acid from the specimen, an ion exchange resin was used. Finally, enzymatic conversion of rhamnose to rhamulose was carried out, followed by the cysteine-carbazole reaction.

An alternative method was also utilized, using ascending paper chromatography to isolate rhamnose (the solvent system consisted of ethyl acetate, pyridine, and water). After drying, the paper was sprayed with aniline hydrogen thalate and oven heated to 105°C. The resultant spots were eluted from the paper with 60% ethyl alcohol and were quantitated by reading the O. D. in a Beckman spectrophotometer at 430 mμ.

Controls which contained all reactants except streptococci were studied. Other controls containing known amounts of intact streptococci free of WBC's were thoroughly hydrolyzed to determine the maximum amount of measurable rhamnose that could be recovered.

Major Findings:

With the enzyme system (rhamnose isomerase) and known L-rhamnose, as little as 1 microgram of rhamnose was detectable. By paper chromatography, rhamnose was separated from all common sugars. Ribose did not travel as far as rhamnose in the solvent system but was close behind. However, when sprayed with aniline hydrogen thalate ribose gave a red spot while rhamnose gave a brown spot. The limit of sensitivity using paper chromatography was 5 micrograms of rhamnose. The results of the experiments in which known amounts of free streptococci were hydrolyzed and total rhamnose was measured by both methods (enzymatic and paper chromatographie), were similar. Studies are now in progress to determine if rhamnose is released in the experimental phagocytic system. As of this date, no definitive results are available.

Significance to Bio-Medical Research and the Program of the Institute:

Phagocytosis and degradation of streptococci are of general interest because of the relationship of streptococcal disease and post-streptococcal sequelae, rheumatic fever and glomerulonephritis. In particular streptococcal cell wall is of interest because this material has been found to persist for long periods of time when injected into animals and is associated with various inflammatory organ lesions.

The chemical events occurring after ingestion of streptococci by phagocytes have been only partially defined. Ayoub and Wannamaker (J. Immunol. 99: 1099-1105, 1967) observed the release of dialyzable components of phagocytized, radioactively labeled organisms and concluded that intraphagocytic digestion did occur. The slow release of dialyzable activity from C^{14} labeled organisms and streptococcal cell wall fractions corresponded to the gradual disappearance of group-specific carbohydrate from whole cells as detected by fluorescent antibody techniques.

In this laboratory the degradation of streptococci by human WBC's has been approached on a morphological and biochemical basis. The latter approach is the subject of the present study. Electron microscopic studies of phagocytosis of streptococci by human PMN's by Ayoub and White (J. Bact. 98: 728-736, 1969) and in this laboratory indicate that these phagocytes may completely digest some streptococci. However, the morphological evidence for cell wall degradation is not unequivocal. Therefore, in order to substantiate that PMN's can degrade streptococcal cell walls a chemical approach assumes importance.

Proposed Course of Project:

The measurements of rhamnose released by phagocytosis and digestion of Group A streptococci by human PMN's will continue, utilizing both enzymatic and paper chromatographic methods.

Honors and Awards: None

Publications: None

Serial No. NIAID 65H1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Morphologic Studies on the Fate of Group A Streptococci Phagocytized by Human Monocytes and Polymorphonuclear Leukocytes.

Previous Serial Number: None

Principal Investigator: Dr. Alan D. Glick

Other Investigators: Dr. Roger M. Cole
Dr. Richard A. Getnick

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	15/12	-	-	15/12
Professional	15/12	-	-	15/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the extent of morphologic degradation of Group A streptococci by human monocytes and polymorphonuclear leukocytes (PMN's) with particular emphasis on the fate of the bacterial cell wall.

Methods Employed:

Group A *Streptococcus pyogenes*, (in M-negative strain derived from Lancefield strain Ti/155) was grown in brain-heart-infusion broth. For use in phagocytosis experiments, exponential phase cultures were suspended in phosphate buffered saline to give a standard reading in a Klett-Summerson Photoelectric Colorimeter of live or heat-killed organisms. An appropriate dilution was then added to monocyte cultures or whole blood.

A modification of the method described by Boyum (Scand. J. Clin. Invest., supplement 97, 77, 1968) was employed to obtain isolated human monocytes. Heparinized venous blood from normal human donors was layered over a mixture of Hypaque and Ficoll and centrifuged, separating the cellular elements of blood into two fractions. The layer containing monocytes and lymphocytes was removed and washed. Moncytes were isolated by allowing them to adhere to tissue culture dishes and subsequently washing them free of lymphocytes. The medium employed was 20% plasma in Hanks Balanced Salt Solution. Log phase streptococci were added to the cell cultures (10 colony forming units per monocyte). After incubation for one hour at 37°C, the cultures were washed to remove extracellular streptococci, incubated in fresh medium at 37°C, and fixed with cacodylate buffered glutaraldehyde at various times. The fixed cells were removed from the culture dishes by scraping with a rubber policeman. Post-fixation in osmium tetroxide, uranylacetate block-staining, embedding, and the preparation of ultrathin sections were done according to standard methods.

To study polymorphonuclear leukocyte phagocytosis, streptococci were added to heparinized blood and incubated at 37°C with constant shaking. Samples were removed at various times, centrifuged in hematocrit tubes, and the buffy coats fixed for electron microscopy as above.

For the preparation of isolated cell walls, streptococci were shaken with 66 μ-diameter glass beads in a Braun disintegrator and separated on sucrose gradients. (Huff, et al, J. Bact. 95: 99, 1968; J. Bact. 88: 1155, 1964)

Major Findings:

Human monocytes readily ingested streptococci, sequestering them within phagocytic vacuoles. Lysis of these ingested streptococci was observed after one hour of incubation. Both the number of lysing organisms and the degree of lysis appeared to increase with increasing incubation time.

Lysis was characterized by decreased cytoplasmic density, increased visibility of mesosomes, reticular changes in the nuclear region, and separation of the cell wall from the cell membrane. Lysis progressed to complete internal disruption with loss of membrane continuity, resulting in a cell wall shell containing only disorganized arrays of membranes and amorphous material. No gross interruptions in cell wall continuity were seen; however, cytoplasmic contents could have been liberated from small breaks in the relatively sensitive cross-wall regions which could not be adequately visualized in tangential sections.

Because of limited survival of monocytes in this phagocytic system, observations could not be extended reliably beyond six hours. At this time there was no evidence that the cell wall was appreciably altered. Only slight reductions in overall thickness were noted (less than $20A^{o}$, compared to controls). The short survival time of the phagocytizing monocytes was shown to be unrelated to the elaboration of leukotoxic factors by the streptococci. Phagocytosis of washed, heat-killed streptococci or latex particles produced the same cellular death.

A previous electron microscopic study of phagocytosis of streptococci by polymorphonuclear leukocytes (Ayoub & White, J. Bact. 98: 728, 1969) suggested that these phagocytes may completely digest some streptococci, but the study was photographically unconvincing regarding cell wall degradation. However, our studies of PMN phagocytosis showed similar results. Many streptococci appeared to lyse, as described above, leaving cell wall shells. However, some rupturing protoplasts could be observed with no attached or adjacent wall fragments, indicating that PMN's may dispose of a limited amount of cell wall material. The walls also appeared to decrease in thickness to a greater extent than in the monocyte study (up to 50A^{o}, compared to control cells).

In spite of these results, there was no unequivocal evidence that either PMN's or monocytes could completely degrade Group A streptococcal cell wall material during the limited time available in in vitro experiments.

Preliminary studies are in progress to determine if PMN's can degrade isolated streptococcal cell walls or mucopeptide shells, prepared by formamide extraction of cell walls. Whole cell walls are resistant to lysozyme hydrolysis while mucopeptide shells are readily lysed. As yet there is no electron microscopic evidence that phagocytes degrade either of these substrates completely.

Significance to Bio-Medical Research and the Program of the Institute:

Previous studies show the rate and pattern of degradation of different species of bacteria after phagocytosis to be variable and dependent on the composition of the ingested organism. Group A streptococci are degraded to a lesser extent than many other bacteria, their cell walls being resistant to intracellular degradation. Numerous studies show that streptococcal cell wall material persists for long periods of time in animal tissue whether isolated walls or whole organisms are injected. These persistent cell wall fragments are associated with chronic inflammatory lesions in several species. Studies by Ohanian and Schwab (J. Exp. Med. 125: 1137, 1967) suggest that these cell wall fragments are released by phagocytic cells. These studies, limited to the light microscope, do not permit assessment of the degree of cell wall destruction within phagocytes, if any does occur.

The present electron microscopic study demonstrates that human peripheral blood monocytes do not have the ability to completely degrade Group A streptococci within six hours in an in vitro system. PMN's appear to have only a limited capacity to degrade these organisms. The cell wall seems to be the resistant moiety. Therefore, it is possible that cell walls persist in migrating monocytes, macrophages, and PMN's in vivo and may be sequestered in tissues where these phagocytes localize, as suggested by studies in rabbits (Ginsburg, et al, Science 166: 1161, 1969). This cell wall material may produce or perpetuate chronic inflammatory lesions through direct toxic effects or hypersensitivity reactions.

In order to determine whether the C-polysaccharide functions to protect mucopeptide from enzymatic hydrolysis, as suggested by Schwab and Ohanian (J. Bact. 94: 1346, 1967), studies of phagocytosis of streptococcal cell walls and mucopeptide shells are in progress.

Proposed Course of Project:

Studies of the phagocytosis and fate of isolated Group A streptococcal cell walls and mucopeptide shells will continue.

It will be determined whether Group A streptococci, cell walls, or mucopeptide shells are degraded by enzyme extracts from human phagocytes and whether chemical determinations can be employed to follow this degradation.

Honors and Awards:

None.

Publications:

None.

Serial No. NIAID 65I
1. Microbiology
2. Bacterial Structure
   and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunology of Streptococcal L Forms

Previous Serial Number: Same

Principal Investigator: Dr. Susan E. Birnbaum

Other Investigators: Dr. Roger M. Cole
Dr. Benjamin Prescott

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4/12	-	12/12	16/12
Professional	2/12	-	12/12	14/12
Other	2/12	-	-	2/12

Project Description:

Objectives:

To study the specificity of antisera made against streptococcal L forms and the role of membrane glycolipids in their serology.

Methods Employed:

Routine methods of bacteriological culture, chemical extractions, ultracentrifugation; standard procedures for immunizing laboratory animals; serological methods such as precipitin tests in capillary tubes and gels, complement fixation, and growth inhibition; thin layer chromatography, solvent extraction, and column chromatography of lipids.

Major Findings:

Rabbits were immunized intravenously with crude membranes made from lysed broth cultures of 5 strains of Group A streptococci and one strain each of a Group D streptococcus, Streptococcus MG, *Proteus mirabilis*, *Staphylococcus* aureus, and Streptobacillus moniliformis. Antisera was also obtained by similar immunizations with parent Group A, C, and D streptococci.

Rabbit antisera against Group A L forms, a Group A parent, a Group D L form and a Group D streptococcus inhibited growth of both Group A and Group D L forms. In addition, when these antisera were tested against lipid extracts (chloroform-methanol 7:3) semipurified by Sephadex G225 column chromatography, they fixed complement and produced positive Ouchterlony tests. Streptococcus MGL antiserum reacted similarly. Antiserum against a staphylococcal L form reacted homologously in all three serological tests but was negative in similar tests against streptococcal L forms. Group C streptococcal antiserum was also negative against other streptococcal antigens.

When antisera were adsorbed with chloroform-methanol 7:3 extracts of either L forms or parent bacteria of Group A or D, there was simultaneous removal of growth inhibiting, complement fixing, and precipitating activity. Adsorption with protein from a delipidated Group A L form with CM 7:3 extracts of the staphylococcal L form failed to remove any serological reactivity from positive antisera. Similarly, adsorption of Groups A and D L forms with CM 7:3 extracts of the staphyloccal L form failed to remove any growth inhibitory activity.

Analysis of the CM 7:3 extract from both L forms and parent streptococci shows that the extracted material is predominantly glycolipid which contains glucose as the only carbohydrate. A cross reaction between Acholeplasma laidlawii and streptococci was observed in growth inhibition tests. This is of interest since A. laidlawii has been shown to contain a similar glycolipid as the serologically active component.

Significance to Bio-Medical Research and the Program of the Institute:

This study shows that antisera against streptococcal L forms or parent bacteria may cross-react within the genus, as measured by complement fixation, growth inhibition or gel precipitation tests. The basis of the common reaction is a glucose-containing membrane glycolipid. Similarly extracted lipid fractions of staphylococci do not cross-react with streptococcal antiserum. The findings suggest that serology may be useful for distinguishing generic origin of L forms, but probably cannot be used to identify L forms within a genus. However, cross-reactions in growth inhibition tests indicate that any serological basis for distinction must be used with care, since a common antigen such as the membrane glycolipid may be present in different genera such as streptococci and mycoplasma.

Proposed Course of Project:

This project is being brought to completion with the termination of the fellowship of the principal investigator. A supply of antisera made against the L forms remains for future study of other antigenic components.

Honors and Awards:

None

Publications: None

Serial No. NIAID 77-1-C
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on *Histoplasma capsulatum*.

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: None

Cooperating Units: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	13/12	0	0	13/12
Professional:	6/12	0	0	6/12
Others:	7/12	0	0	7/12

Project Description.

Objectives:

To determine the effects on the viability and recoverability from soil of *H. capsulatum*, of changes in the soil microflora.

Methods Employed:

Studies of the viability of *H. capsulatum* in autoclaved and raw soil were continued. The total populations of streptomycetes and bacteria in soil samples that were positive or negative for *H. capsulatum* were determined. The effect of the addition of bat guano on the survival of *H. capsulatum* in a microflora composed of a combination of various bacteria and streptomycetes isolated from the soil negative for *H. capsulatum* were studied.

1) Soil samples were collected from 8 different sites from Clarksburg, Maryland in April, 1969. Portions of each sample was tested for the presence of *H. capsulatum*. The rest of each sample was divided into two equal parts in Erlenmeyer flasks. One portion of each sample was autoclaved for one hour at 120°C on 2 consecutive days. The other portion of the samples was not treated. A thick suspension of spores and mycelium was prepared using a typical strain of *H. capsulatum*. Each soil sample was inoculated

with the fungal suspension and incubated at 25°C. The test for the presence of H. capsulatum was made every 6 months by the mouse passage technique.

2) A soil infusion was prepared by autoclaving 7000 g of soil from Clarksburg suspended in 7 liters of water for a total of 15 hrs. The muddy sterile infusion was dispersed (250 cc each) into 500 ml flasks. These infusions were inoculated with various levels of spore suspensions of ten streptomycetes strains and cell suspensions of ten bacterial strains isolated from 4R sample (negative for H. capsulatum). Filter sterilized bat guano infusion was added to one series of flasks. Conidia of H. capsulatum were added to each flask and incubated on rotary shaker at room temperature. The samples were removed periodically and injected into mice as well as plated on various selective media for the different groups of microorganisms. The media used for culturing each group of organisms were: 1) brain heart infusion agar (BHI) for bacteria, 2) starch agar, 400 mg/l cyclohexamide plus 1 g/l furacin for streptomycetes and 3) soil extract agar plus 100 mg/l gentocin for H. capsulatum. The mice were autopsied 3 weeks after the injection of each sample and the organs were cultured on both BHI with antibacterial antibiotics and soil extract agar with gentocin.

Major Findings:

A). H. capsulatum survived well for at least 2 years when inoculated into autoclaved soil whether the soil was originally positive or negative for this fungus. In the raw soil, however, when H. capsulatum was added, it survived only when the soil was originally positive. Most of the raw soils from the original H. capsulatum negative site yielded no cultures of H. capsulatum 18 months after inoculation. The pH of the original raw soil varied from 4.6 to 7.5 and no correlation was found between the pH and the presence of H. capsulatum.

The raw soil from the sites originally positive for H. capsulatum contained markedly fewer streptomycetes than the soil from the negative sites. Soils containing the highest population of streptomycetes were not only originally negative for H. capsulatum but H. capsulatum did not survive for six months after inoculation. The soils originally positive for H. capsulatum contained markedly more bacteria than streptomycetes when compared to the soils negative for H. capsulatum. Although originally negative for H. capsulatum, the added fungus survived well if the raw soil contained considerably less streptomycetes than bacteria.

B). The soil infusion containing various streptomycetes, bacteria and a filtrate of bat guano supported the growth of H. capsulatum better than the identical soil infusion without bat guano. Up to three weeks after inoculation of H. capsulatum, there was a discrepancy between the isolation of H. capsulatum by mouse passage technic and by direct plating. There was good correlation in the results between the two methods of isolation from 5 weeks after inoculation. The number of H. capsulatum isolated from all the soil infusions with bat guano 9 weeks after inoculation of the fungus had increased drastically, while most of the soil infusions without bat guano yielded no H. capsulatum. The bacterial population increased much faster in the series of soil infusions with bat guano than those without bat guano. The population of streptomycetes, however, increased slightly over 9 weeks in the series with bat guano while almost no change was detected in the series without bat guano.

Significance to biomedical research and the program of the Institute:

This study provides valuable information concerning the establishment and viability of H. capsulatum in its natural habitat, the soil. It will also furnish some information on the relationship between the growth of H. capsulatum and other microflora such as actinomycetes and bacteria in soil. Soils contaminated with bat or bird guano have been known as the better environment for the existence of H. capsulatum. This project will provide some information on effect of bat guano on the survival of H. capsulatum in these simulated natural conditions.

Proposed course of project:

The project will be expanded to include field studies. The change of streptomycete and bacterial populations after the addition of bat guano will be analyzed. Efforts will be made to identify streptomycetes producing antifungal (especially anti H. capsulatum) antibiotics in soil.

Honors and Awards.

None

Publications.

None

Serial No. NIAID 77-1-E
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the pink mutants of *Candida albicans*.

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigator: None

Cooperating Units: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	0/12	-	-	0/12
Professional:	0/12	-	-	0/12
Other:	0/12	-	-	0/12

Project Description.

See 1970 Annual Report.

Project Terminated.

Serial No. NIAID 77-1-F
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on *Aspergillus bisporus*.

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4/12	0	0	4/12
Professional:	2/12	0	0	2/12
Other:	2/12	0	0	2/12

Project Description.

Objectives:

To investigate the manner of kidney invasion by two different types of spores produced by the newly described fungal pathogen, *Aspergillus bisporus*. The path by which the large spores reached the pelvic space before germination was also investigated.

Methods Employed:

Mice were injected intravenously with primary and secondary spore suspensions (each $2x10^7$ spores) and sacrificed at designated time intervals. Histological tissue sections of kidney were prepared.

Major Findings:

Although the *in vitro* environmental conditions required for the strains of *A. bisporus* to produce the small secondary spores are different from the large primary spores, the manner of kidney invasion by the two types of spores was identical. Kidneys removed 1 and 2 hours after the infecting dose was administered showed spores only in glomeruli. Sections prepared 4, 6 and 22 hours after injection showed spores in both glomeruli and outer cortex. Sections prepared after 4 and 9 days showed that some spores had reached the inner cortical area. The sections obtained at 2 and 3 weeks showed no fungal cells in the cortex or Bowman's space, but extensive hyphal growth was observed in the tips of papillae and in the pelvic area. The host defenses appear to have removed the organism from the renal tissues by this time except in the lumen of the extreme distal ends of the collecting tubules, thereby causing necrosis in the papillary tips. The only evidence of cortical damage was minimal scarring and subacute inflammation around the arcuate vessels. Interstitial capillaries near the tip of papilla were engorged with platelets. Although the primary spores are larger than red blood cells, the spores seem to have gained entrance into the tubules by passing through the glomerular capillaries into Bowman's space. Since the fungus was not found in the cortex 2 weeks after injection, it seems unlikely that the lesions in the area resulted from ascending infection from the pelvis.

Significance to biomedical research and the program of the Institute:

Aspergillus bisporus is a newly described pathogenic fungus causing severe, chronic kidney infection of mice. Unlike other fungal pathogens primarily invading the kidney in mice, the cells of *A. bisporus* invade and multiply only in the lumen of the extreme distal ends of the collecting tubules and in the pelvic space. The study of pathogenicity of the organism in the kidney of animals will contribute knowledge towards understanding of certain infectious processes, especially those caused by organisms larger than red blood cells.

Proposed Course of Project:

The project will continue using inbred mice with primary polydipsia, STR/N, to study the effect of low osmolarity and specific gravity of urine on the pathogenicity of *A. bisporus*.

Honors and Awards.

None

Publications.

Kwon-Chung, K. J., Fennell, D. I.: A new pathogenic species of *Aspergillus*. Mycologia. (In press).

Kwon-Chung, K. J., and Hill, W. B.: Studies on the pink, adenine-deficient strains of *Candida albicans*. I. Cultural and morphological characteristics. Sabouraudia. 8:48-59, 1970.

Serial No. NIAID 77-1-G
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genetic Study of _Blastomyces dermatitidis_.

Previous Serial Number: None

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: None

Cooperating Units: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	7/12	0	0	7/12
Professional:	4/12	0	0	4/12
Other:	3/12	0	0	3/12

Project Description.

Objectives:

To continue investigation of genetic control of the mating system in _Ajellomyces dermatitidis_ (_Blastomyces dermatitidis_).

Methods Employed:

Strains ATCC 18187 (A= + type) and ATCC 18188 (a = - type) were crossed on soil extract agar. The F_1 progeny derived from the single ascospores were converted to the yeast form on blood-glucose-cysteine agar slants at 37°C. The F_2 progeny were also obtained by crossing compatible strains selected among the F_1 strains. These were paired with the yeast phase of parent strains or sib-crossed in all possible combinations on soil-extract agar plates and incubated at room temperature to revert to the mycelial form. The 40 strains of old and recent human isolates from the United States and Africa maintained on Sabouraud glucose agar slants for various lengths of time were also tested for their mating type.

Major Findings:

The 40 F_1 progeny obtained by isolating single ascospores showed 1:1 ratio for buff _vs_ white colonies. All the buff strains were fertile in back-crossing with one or the other parent and 50% fertile in sib-crossing. The white strains were sterile in back-crossing and 25% fertile in sib-crossing. However, stimulation of aerial growth was noted along the line of contact with either one or the other parent. The microscopic examination of both white and buff strains indicated that the loss of pigment is not due to the loss of asexual sporulation as occurring in other pathogenic fungi but due to the loss of pigment within the mycelium and conidia. Two opposite mating types of highly fertile, buffy F_1 strains were crossed and 26 F_2 progeny were obtained. It was found that all 26 were pigmented and fertile in back-crossing. The ratio between + and - types was 1 (11): 1 (15). Thirty F_2 progeny were also isolated by crossing two opposite poorly fertile, lightly buff F_1 strains. Of these 30 F_2 strains, 6 strains were white and sterile in back-crossing. Among the remaining 24 strains 10 crossed with a + parent and 14 crossed with a - parent. The quantity of cleistothecia produced in this group was markedly less when compared to the F_2 strains from buffy, highly fertile pair. Examination of 18 clinical isolates which were maintained on agar media for more than 2 years showed that only 11% retained their mating competence and pigment while of 22 fresh clinical isolates, maintained less than one year, all but 1 produced pigment and mated with tester strains. These results indicate that the mating in _Blastomyces dermatitidis_ is controlled by a bipolar incompatibility system in which the progeny of a single fruiting body are of two mating types and that pigmentation in a colony is directly related to the competence of fruiting in the sexual stage.

Significance to bio-medical research and the program of the Institute:

This study indicates that there are only two mating type strains among the organism causing North American Blastomycosis. Fruiting competence of the perfect state in _Blastomyces dermatitidis_ is found to be directly related to the formation of buffy pigment in each strain and is independent of the mating factors. Deterioration of the fruiting competence and pigment formation is extremely rapid in this species when maintained in the laboratory by conventional method. When the mating type test for an isolate is necessary to confirm the identification of the organism for best results, the test should be performed within a short period of the isolation.

Proposed Course of Project:

The project will be pursued to determine 1) the genetic control of dimorphism and 2) the variability in mating competence among the F_1 strains maintained by _in vitro_ and _in vivo_ methods.

Honors and Awards.

None

Publications.

Kwon-Chung, J. K.: Genetic Analysis on the Incompatibility System in _Ajellomyces dermatitidis_. Sabouraudia. 9. (In press).

Serial No. NIAID 78-A
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Experimental Therapy of Mycoses

Previous Serial Number: Same

Principal Investigator: George W. Lones

Other Investigators: Terry Popkin, Benjamin Prescott

Cooperating Units: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	19/12	0	0	19/12
Professional:	7/12	0	0	7/12
Other:	12/12	0	0	12/12

Project Description.

Objectives:

To test the toxicity and efficacy of new antibiotics and chemicals against experimental mycoses in mice and other animals and to investigate such drugs preliminary to clinical trials. To seek synergistic and additive effects in combinations of antimycotic drugs and antibiotics.

Methods Employed:

Over a period of several years a standard method of testing drugs in mice which is efficient and informative has been developed. Mice are infected intravenously with a carefully estimated infectious dose of fungus cells which kills control (untreated) mice in 14-21 days. Treatment is begun on the third post-infection day and treated mice are given the test drugs at dose levels up to tolerance. Effectiveness of the drug is measured in terms of its ability to delay or prevent death. At the termination of the test, the effectiveness is further measured by its ability to clear tissues of the pathogenic fungus in treated mice which have survived the experiment. Drugs and antibiotics are obtained from various sources after the contributor has supplied evidence of alleged or probable efficacy. Dr. Benjamin Prescott has synthesized drugs of series which have for various reasons offered promise of usefulness. These are examined for ability to inhibit the growth of systemic fungi *in vitro* before consideration in *in vivo* evaluation.

Major Findings:

Bayer 5097 is a synthetic compound which has been reported to be effective in human candidiasis. Trials in which this agent was administered orally to mice with experimental coccidioidomycosis indicated that Bayer 5097 possessed some therapeutic activity. When administered at the rather high dosage rate of 200 mg/kg/day, the drug was capable of preventing death of a significant number of otherwise lethally infected mice.

Five experimental antifungal agents prepared by E. I. du Pont de Nemours & Co. were tested for *in vitro* activity against five systemic fungal pathogens. All the compounds exhibited moderate activity. Two were selected for evaluation in Histoplasma-infected mice, but no significant therapeutic activity was demonstrated.

5-Brome indol-3-carboxaldehyde salicyladehyde hydrazone, prepared by Dr. Prescott, capable of inhibiting *H. capsulatum* at low concentration *in vitro* did not exhibit therapeutic activity in this infection in mice.

Fifty derivatives of p-methoxybenzylhydrazino carboxylate and a similar number of furfural hydrazone derivatives were tested for *in vitro* activity against *H. capsulatum* and *Cryptococcus neoformans*. None was considered sufficiently active to warrant *in vivo* trials.

A study of the effect of growth of *H. capsulatum* in the presence of the antibiotic saramycetin on the fine structure of this fungus was continued. A by-product of this study was the observation that some strains of *H. capsulatum* contain clusters of intra nuclear bodies not previously described. These structures, round to slightly oval in section, and averaging about 50 nm. in diameter have been seen in two of four strains of *H. capsulatum* yeast cells examined. Their nature is not yet known.

Significance to biomedical research and the program of the Institute:

Amphotericin B and hamycin are the only antimycotic antibiotics now generally available and they are quite toxic. It is essential to encourage the discovery and development of new therapeutic agents for the systemic mycoses.

Proposed course of project:

The project will be discontinued due to retirement of the principal investigator.

Honors and Awards.

None

Publications.

Lones, G. W. and Popkin, T.: Effect of Saramycetin on the Fine Structure of Histoplasma capsulatum. Mycopathologia et Mycologia Applicata. In press.

Serial No. NIAID 78-B
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Physiology of *Coccidioides immitis*.

Previous Serial Number: Same

Principal Investigator: George W. Lones

Other Investigators: None

Cooperating Units: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	16/12	0	0	16/12
Professional:	6/12	0	0	6/12
Other:	10/12	0	0	10/12

Project Description.

Objectives:

To obtain information on the physiology, nutrition and metabolism of *C. immitis*, especially as these factors relate to its dimorphism.

Methods Employed:

Shake culture techniques are employed to produce the parasitic and saprophytic morphologic modifications of several strains of *C. immitis* in adequate quantities for examination by chemical and biochemical techniques. Washed cell suspensions are used for respiration studies. Cell-free extracts are prepared by disruption of cells in a frozen state at high pressure. Enzymatic activities are determined by chemical and biochemical procedures employing such techniques as the Warburg apparatus, spectrophotometry, chromatography and ionophoresis. Factors influencing growth rates of cultures are evaluated by determining the weights of mycelium or spherules produced under defined conditions. The interconversion of the two forms is studied by direct microscopic counts of the number of mycelial particles formed in spherule cultures. The effects of culture conditions, composition of the medium and various supplements on the transformation are studied.

Major Findings:

Major efforts were directed to identifying and measuring the effect of stimuli to reversion of culture spherules to mycelium. The marked effect of small concentration of serum proteins previously noted was studied further. Although protein hydrolysates at concentrations comparable to the concentration of serum proteins are also active, the activity of serum does not appear to be dependent upon proteolysis by the spherules. About 90% of added serum proteins can be accounted for in culture filtrates following incubation, and there is evidence that some of the protein is precipitated and removed by the filtration. The activity of protein hydrolysates, such as casein hydrolysate is approximately equaled by a synthetic mixture of L-amino acids based on the amino acid composition of casein. The activity of the amino acid mixture is not due to any one of the component amino acids, but appears to result from the combined action of the components of the mixture.

Significance to Bio-medical Research and the Program of the Institute:

Coccidioidomycosis is a health problem in certain areas of this country. Methods available for the treatment of the progressive form of the disease are far from satisfactory. A better knowledge of the causative agent may favorably influence our diagnosis, prevention and treatment of the infection.

Proposed Course of Project:

The project is to be discontinued due to retirement of the principal investigator.

Honors and Awards.

None

Publications.

None

Serial No. NIAID 79-B-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Serologic Studies of Leukemic Patients, using Fungal Antigens.

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: William O. Mitchell

Cooperating Units: Leukemia Service, Medicine Branch, NCI (Dr. Geoffrey Herzig)

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	7/12	0	0	7/12
Professional:	4/12	0	0	4/12
Others:	3/12	0	0	3/12

Project Description.

Objectives:

To study the antibody response to fungal infection in leukemic patients and its application to laboratory diagnosis.

Methods Employed:

Serum samples are tested for antibodies to antigens of *Candida albicans* antigenic group A. Heat-killed whole yeast cells are used as antigen for agglutination, a soluble extract (S) from sonically disrupted viable yeast cells and mannan (M) extracted from yeast cells are used as antigens in immunodiffusion. For agglutination a fourfold rise in titer is interpreted as a positive response. The observation of a precipitin band with either soluble antigen is interpreted as a positive reaction. A rise in titer of agglutinins is more frequently observed.

Major Findings:

Earlier studies on the serology of aspergillosis have been discontinued. Although a precipitin titer of 1:8 with a polysaccharide produced by Aspergillus fumigatus was shown to be correlated with tissue invasion by this fungus, these precipitin reactions were found to be a measurement of C reactive protein. No antibody response to antigens of A. fumigatus has been shown in leukemic patients.

Eighteen of 91 uncultured leukemic patients have shown an antibody response to antigens of Candida albicans. Thirteen of the patients had both precipitins and agglutinins. Using a standard concentration (1 mg/ml) of either soluble antigen in immunodiffusion, the sera of only 7 patients precipitated, but when serial twofold dilutions of the antigens were made, sera from six more patients reacted with the higher dilutions. The lowest agglutinin titer of any serum that reacted in immunodiffusion was 1:160 but all others had agglutinin levels of 1:320 or 1:640. Four of the five patients whose sera did not precipitate had maximum agglutinin titers of 1:320 or 1:640. Using multiple dilutions of the soluble antigens did not result in the demonstration of precipitins in these sera.

Significance to biomedical research and the program of the Institute:

Serology augments other laboratory procedures for the diagnosis of candidiasis in immunologically compromised patients.

Proposed Course of the Project:

To continue in order to collect more information concerning the interpretation and limitations of the tests.

Honors and Awards.

Presentations:

Serological investigations of candidiasis. Seminar, Temple University, Jan. 21, 1971. H. F. Hasenclever.

Present serological techniques for the diagnosis of fungal disease. Seminar, Veterans Administration Hospital, Washington, D. C., April 8, 1971. H. F. Hasenclever.

Member, Examining Committee for Certification in Medical Mycology, American Board of Microbiology. H. F. Hasenclever.

Publications.

Preisler, H. D., Hasenclever, H. F., Henderson, E. S., Smith, J. K., and Louria, D. J.: A prospective study of circulating anti-Candida substances in leukemic patients. J. Infectious Diseases. (In press).

Preisler, H. D., Hasenclever, H. F. and Henderson, C. S.: Anticandida Antibodies in Patients with Acute Leukemia. Amer. J. Med. (In press).

Serial No. NIAID 79-C-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ecologic and Aerobiologic Studies of *Histoplasma capsulatum*.

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: Willard R. Piggott

Cooperating Units: None

Man Years

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	21/12	0	0	21/12
Professional:	4/12	0	0	4/12
Other:	17/12	0	0	17/12

Project Description.

Objectives:

To study the factors that contribute to the presence or absence of *Histoplasma capsulatum* in soil.

Methods Employed:

The principal sites under study are at Clarksburg, Maryland and at 7th Street and Pennsylvania Avenue, Washington, D. C. Soil samples are collected at intervals and the absence or presence of the fungus is ascertained by mouse inoculation.

Major Findings:

Results of soil sampling at Clarksburg, Maryland, indicate that in a defined area the presence and numbers of *Histoplasma capsulatum* has remained unchanged for a period of 12 years. The fungus has not extended its zone of residence nor has it been reduced. Abandonment of the site and destruction of the house in 1966 has resulted in the overgrowth by herbaceous annuals and perennials and woody perennials. To date this change of flora has not affected the presence of *H. capsulatum*. In another defined zone at this site (one which is weakly positive), 3 applications of bat guano over a period of 18 months has not resulted in a detectable increase in the presence of the fungus.

Studies at the other site, 7th Street and Pennsylvania Ave., indicate little change in the presence and numbers of H. capsulatum over the last 3 years. There was however a drastic diminution of the fungus from 1963 to 1966.

The differences between the two sites justify comment. The one at 7th and Pennsylvania was formerly a roost for starlings, whereas bats were inhabitants in the house at Clarksburg. Four years after the roost for starlings was destroyed, the presence of H. capsulatum had declined, although at Clarksburg 4 years after the bat roost had been removed, the fungus has neither extended its colonization in the soil or receded. The most apparent difference between the sites is the care they receive. At 7th and Pennsylvania, the site, under the care of the National Park Service, receives intensive lawn conditioning and the shrubs are heavily mulched, whereas at Clarksburg the site is overgrown and returning to a natural state.

Significance to biomedical research and the program of the Institute:

This study is providing information concerning the longevity of H. capsulatum in two different environments.

Proposed Course of Project:

To continue studying the effect of transposition of a unit of positive soil to a negative area and to an area that is negative but has been fertilized with bat guano; and to determine the effect of a heavy application of mulch to a positive area.

Honors and Awards.

H. F. Hasenclever - Member, Advisory Panel for Aerobiology Program, United States National Committee, International Biological Program.

Presentation: Histoplasma capsulatum: its free living state. Seminar, Temple University, Jan. 21, 1971. H. F. Hasenclever.

Publications.

None.

Serial No. NIAID 79-D
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunochemical Studies on Polysaccharides from Fungi

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: William O. Mitchell

Cooperating Units: None

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	15/12	0	0	15/12
Professional:	12/12	0	0	12/12
Others:	3/12	0	0	3/12

Project Description.

Objectives:

To study the molecular structure, serologic properties, and the haptenic groups of polysaccharides isolated from yeasts and fungi.

Methods Employed:

Polysaccharides are extracted from fungal cells or isolated from the culture media in which the fungi were grown. Acidic or neutral polysaccharides are separated by gel chromatography and their serologic activity studied by immunochemical techniques. Antisera are prepared by the injection of donor rabbits with fungal cells.

Major Findings:

Previously it was believed that mannans isolated from Candida species were neutral. Recent studies have shown that most of these mannans contain an acidic polysaccharide in addition. Immunochemical reactions indicate that these acidic mannans are structurally related to similar polysaccharides produced by Hansenula species. Recent studies also indicate that the composition of medium is unrelated to the type of polysaccharide produced.

Significance to biomedical research and the program of the Institute:

This work has shown that there is correlation of structure to immunochemistry of mannans but many problems are to be solved in order to define this correlation. More knowledge of these antigenically active polysaccharides will elucidate some of the problems now present in the serological techniques used in diagnosing some of the fungal caused diseases.

These studies are defining the antigens that provoke the antibody responses in some fungal infections.

Proposed Course of Project:

To continue attempting to induce antibodies against some of these polysaccharides in rabbits and studying the newly isolated acidic and neutral polysaccharides immunochemically.

Honors and Awards.

None

Publications.

None

Serial No. NIAID 79-E
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Yeast Flora of Birds

Previous Serial Number: None

Principal Investigator: H. F. Hasenclever

Other Investigators: None

Cooperating Units: Patuxent Wildlife Research Center, Dr. Richard Kocan.

Man Years.

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	5/12	0	0	5/12
Professional:	4/12	0	0	4/12
Other:	1/12	0	0	1/12

Project Description.

Objectives:

To study the yeast flora of columbifora birds and to compare the yeasts isolated with the same species of yeasts that are known to inhabit and cause disease in man.

Methods Employed:

The throats of live or recently sacrificed birds are swabbed and the swabs used to inoculate neopeptone glucose agar plates. In some cases contents of the crop are also used as inocula. Plates are incubated at 37°C and after 24 or 48 hours all the different colonial types of yeasts are picked and subcultured on agar slants. The yeasts are then identified using morphological and physiological characteristics. Lodder, J. (Ed.) The Yeasts - a taxonomic study (Ed. 2). Amsterdam, North Amsterdam Publishing Company, 1970, 1385 pp is the reference used in identification.

Major Findings:

From 44 common pigeons, Columba livia, 45 isolations of yeasts were made. Twenty-eight were Candida albicans, 2 C. tropicalis, 3 C. krusei, 4 Torulopsis glabrata, 6 Saccharomyces telluris, and 2 Geotrichum species.

C. albicans and C. krusei were both isolated from one bird. Twenty-five strains of C. albicans belonged to antigenic Group A and 3 to Group B.

Nine mourning doves, Zeraidura macroura, have been cultured. C. albicans was isolated from 6 birds, C. tropicalis 1, C. guilliermondii 1, and Geotrichum species from 1. All strains of C. albicans belonged to antigenic Group A.

White-crowned pigeons (Columba leucocephala), a tropical species whose northern most range is the keys of Florida, were studied. A single species of yeast, S. telluris, was isolated from each of the 23 birds cultured. Of probable ecologic significance is that the optimal temperature for growth of this microorganism is from 37° - 40° C., and that below 30° C. its growth is sparse. The strains of this yeast that were isolated from the common pigeon differ antigenically from those isolated from the white crowned pigeon. These isolates are not pathogenic to mice.

C. tropicalis, when incubated initially at 16°, 25°, 30°, or 37° C and finally at 5 - 9°C produces terminal forms similar to chlamydospores that are produced by C. albicans. This unique response to temperature was shown to be consistent in all 28 strains studied.

Significance to biomedical research and the program of the Institute:

These results extend our knowledge concerning the ubiquity of some of the opportunistic yeasts and their existence in species of this family of birds. The strains isolated appear to be identical morphologically, physiologically, and antigenically with those isolated from human beings. These yeasts are known to inhabit many birds and animals indicating their biologic versatility and importance as potential pathogens among diverse homoiothermic species.

Proposed Course of Project:

To continue studying the yeast flora of more species of columbifora birds and to investigate the nature of the antigenic differences observed among the isolates of S. telluria and their antigenic relationships with Candida and Torulopsis species.

Honors and Awards.

None

Publications.

Hasenclever, H. F.: A unique condition required for the consistent production of chlamydospores by Candida tropicalis. Sabouraudia, in press.

Serial No. NIAID-207
1. Microbiology
2. Mycoplasma
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Basic Studies on Mycoplasma

Previous Serial Number: Same

Principal Investigator: Joseph G. Tully, Ph.D.

Other Investigators: None

Cooperating Units: Laboratory of Bacterial Products, DBS (DBS-69-59)
Laboratory of Parasitic Diseases, NIAID (NIAID-123B)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	60/12	-	-	60/12
Professional	12/12	-	-	12/12
Other	48/12	-	-	48/12

Project Description:

Objectives:

The objectives of these studies cover both basic and applied aspects of Mycoplasma including their nutrition and metabolism, mechanisms of virulence, immunological interrelationships among human and animal strains, and their role in human infection and certain other mixed bacterial, viral and protozoan diseases of man and animals.

Methods Employed:

Recovery of mycoplasmas from various hosts was accomplished by standard cultivation techniques and all agents were serologically typed by fluorescent antibody procedures. Characterization of sterol-nonrequiring mycoplasmas was made on the basis of biochemical and serological properties and on cell protein patterns obtained by gel electrophoresis. Animal mycoplasmas were compared in a variety of biological and serological procedures to other prototype human and animal Mycoplasma strains in the current culture collection. The gel electrophoretic pattern of Mycoplasma cell proteins was also employed in a study of various techniques useful in rapid identification of Mycoplasma serotypes.

Major Findings:

Earlier investigations (see prior annual report) concerned several mycoplasmas capable of growing in a sterol-free environment and all were serologically distinct from the single Mycoplasma serotype heretofore known to lack a sterol requirement. The observations led to recent taxonomic changes to reclassify the sterol nonrequiring mycoplasmas as Acholeplasma species. We have characterized and described one of the new serotypes as Acholeplasma axanthum. Current studies now indicate that the acholeplasmas may represent a much larger group of distinct species than originally thought. Two additional mycoplasmas under active investigation now appear to possess properties similar to those of other acholeplasmas. One strain was recovered from swine while the other strain was initially recovered from a tissue culture line. Both cultures were examined in the sterol requirement test previously developed in these studies and each was shown to lack a requirement for cholesterol. There was no evidence that the strains possessed pigmented carotenoids and each was serologically distinct from the other acholeplasmas by growth-inhibition and epi fluorescent antibody tests. Electrophoretic analysis of their cell protein patterns have shown some similarity but not complete identity with the patterns of A. axanthum strains. Further analyses of the strains by reciprocal growth-inhibition tests and other serological tests are in progress to establish their ultimate relationship to other sterol-nonrequiring mycoplasmas. Several other mycoplasmas of bovine origin have been received and preliminary examination suggests they may also fall into the Acholeplasma genus. The frequent occurrence of bovine mycoplasmas in tissue culture systems fed with fetal calf serum may point to the origin of some of the other acholeplasmas that have been isolated from cell cultures.

Further characterization of new monkey mycoplasmas reported here last year has confirmed the relationship of a large number of these isolates to an unclassified Mycoplasma (strain Navel). The latter strain was originally recovered from a human umbilical lesion and was formerly thought to represent a mycoplasma of human origin. Although many of the mycoplasmas recovered from monkeys also appear to be associated with man and other primates, the Navel strain would now appear to be predominately a part of the normal flora of monkeys. We have carried out extensive comparisons between various monkeys isolates related to strain Navel and between Navel cultures carried in other laboratories. The cell protein patterns of these strains by acrylamide gel electrophoresis indicates that they are closely related. Additional tests on their sterol requirements, biochemical properties, and serological characteristics confirm this relationship. In cooperation with other collaborators, these strains are being proposed as a new species (Mycoplasma primatum).

Additional studies have also been carried out on a group of eight mycoplasmas recovered from a variety of animal hosts including sheep, goats, bovines, swine, and a single human isolate. All of these mycoplasmas show some relationships to several pathogenic goat mycoplasmas. A variety of serological and biochemical tests have been performed to establish their interrelationships. All of the mycoplasmas are carbohydrate fermenting strains and several also hydrolyze arginine. Many of them also possess strong proteolytic properties as evidenced by their rapid liquefaction of coagulated serum. Gel electrophoresis studies have shown a basic similarity in the cell protein patterns, particularily in the lower portions of the gel, but some differences are apparent in other parts of the gel pattern. Serological analysis has included extensive reciprocal testing with the growth-inhibition, epi fluorescent antibody, immunodiffusion, complement-fixation, and metabolic inhibition procedures. Our findings, at present, do not provide clear evidence that any one of these serological tests will show that all of the strains are serologically similar. The metabolic-inhibition test appears to provide the best information that these strains are related. There is some evidence that this heterogeneity is associated with the presence of some soluble membrane antigens and that these antigens might be partially lost or degraded when strains are grown in liquid media. Antisera prepared with these degraded antigens would thus fail to possess complete antibody necessary to relate the strains through most serological tests. Currently, procedures to prepare antisera without the loss of soluble antigens have been successful and the resulting antisera are under test.

Studies continue on the role of mycoplasmas, particularly M. neurolyticum, in a paralytic syndrome in rats with an experimental Plasmodium berghei infection. Additional investigations were carried out on mycoplasmas recovered from the spleen of rats inoculated at various times with the malarial strain being carried in splenectomized rats. It was anticipated that the passage of the paralysis-inducing malarial strain in this type of host might alter the relationships between the suspected mycoplasmas and host so that mycoplasmas could be recovered in culture. Mycoplasmas were recovered from rat spleens with this procedure and five of the strains were typed as species of M. iners, a serotype commonly found in avian hosts. The isolation of this non-paralytic Mycoplasma serotype from different rats is not readily explained at present. Two additional mycoplasmas were also recovered but it has not been possible to type them as recognized Mycoplasma species. Efforts were also renewed to isolate mycoplasmas from rats with mixed infection by passing blood from paralyzed rats through germ-free mice. Germ-free mice were selected over conventional mice since the latter frequently carry latent mycoplasmas. The initial attempt was carried out with a single passage of rat blood to 10 germ-free mice. Two of the germ-free mice showed some evidence of neurological disease but both died before adequate culture tests could be performed. Tissues of remaining animals were subjected to extensive culture but did not yield mycoplasmas. From earlier observations and some current tests in conventional mice, it was evident that at least two mouse to mouse passages of rat blood were required to produce a consistent paralytic syndrome in mice. Attempts to carry out repeated passage of rat blood in germ-free mice are in progress and it is anticipated that this procedure will ultimately be successful in demonstrating M. neurolyticum in the mixed malarial infection in rats. In an additional effort to provide confirmation of the role of this Mycoplasma species in the paralytic syndrome, a repeat of the partially successful passive protection test in rats using antibody prepared against the neurotoxin of M. neurolyticum is in progress. This study will entail the use of more potent antitoxin combined with its more frequent administration to rats over the early stages of the malarial infection. The ability of the neurotoxin antibody to suppress the paralytic disease should provide further evidence of the role of M. neurolyticum in this syndrome.

The fluorescent antibody test using agar-grown mycoplasmas and conjugated type-specific antiserum has received extensive use this year in typing tissue culture isolates. A large number of mycoplasmas recovered from primary mouse tissue cultures and other cell lines have been examined. The plate fluorescent test has shortened the time required for typing and has conserved significant quantities of materials normally required to grow sufficient amounts of antigen for identification purposes. Typing of mycoplasma contaminants in tissue culture systems can provide important information on possible sources of the contamination and indicate whether primary tissue mycoplasmas are involved or exogenous cell culture materials are contaminated. As new Mycoplasma strains and serotypes are identified, we have prepared conjugated antisera to each and carried out serological comparisons of other mycoplasmas in our collection. In several instances we have shown that strains named as new species by others were actually related to previously established species (e.g. M. leonis recovered from the lung of a lion was typed as M. arginini). Several observations have also been made on improving the quality of the conjugated antisera. We have noted that there is a direct correlation between the ability of an antiserum to inhibit the growth of mycoplasmas (disc inhibition) and its fluorescent antibody titer. Thus, immunization procedures are continued until each individual antiserum possess disc inhibition zones of 6 mm or more before it is conjugated. Some mycoplasmas do not readily produce satisfactory growth-inhibition antiserum and various methods to improve the usual techniques have been carried out this year. Preliminary results indicate that the inoculation of rabbits with mycoplasmal broth cultures mixed with adjuvant gives superior results. Both foot pad and intramuscular inoculations are combined for the best results. The gel electrophoresis of mycoplasmal proteins continues to provide important confirmation of the biochemical and serological properties of these organisms. Gel patterns have been obtained on most mycoplasmas in our collection and comparisons are now being made on groups of species with similar quanine/cytosine ratios. Those species with similar electrophoretic patterns and nucleic acid base composition values may yield important findings in nucleic acid hybridization studies.

Significance to Bio-Medical Research and the Program of the Institute:

The unique host specificity originally described for mycoplasmas is rapidly changing as new information on techniques and the basic biology of this group of microorganisms unfolds. As a consequence of this new information and to determine adequately the role of mycoplasmas in human diseases and in diseases of undetermined etiology, the biological and serological characteristics of all human and animal mycoplasmas must be more adequately known and described. Studies on the sterol nonrequiring mycoplasmas can provide some important information on methods by which microorganisms can synthesize cholesterol and other essential lipids required for the structure and integrity of the limiting membrane. Although the presence of pigmented carotenoid compounds in the cell membrane of some sterol nonrequiring mycoplasmas was originally thought to play a part in their ability to grow in the absence of sterols, the absence of carotenoids in the new acholeplasmas casts doubt on this theory. The makeup of sterols in the cell membranes of the new species is, therefore, of considerable interest to an understanding of lipid biosynthesis. Studies on new animal mycoplasmas of human origin, as well as those mycoplasmas associated with mixed experimental infections, can help delineate the role of these organisms in disease. Technical aids that shorten the time necessary to separate and identify mycoplasmas are of an applied or diagnostic value.

Proposed Course of Project:

Further studies are in progress on human and animal mycoplasmas and their importance to man. These studies are particularly directed to three points:

1. Are some human diseases of undetermined etiology related to animal mycoplasmas?
2. Can some mycoplasmal diseases in animals provide appropriate models for the study of the role of these agents in human diseases?
3. Are there techniques useful in demonstrating latent mycoplasmas in animal hosts that can be applied to some human diseases where mycoplasmas might be involved?

Some of the critical factors in studies of this nature involve questions about what component in mycoplasmas is responsible for virulence and how this component can be maintained in artificial systems. Attempts to learn more about mycoplasmal neurotoxin is in progress as well as those procedures that might reveal latent mycoplasmas in various animal hosts.

Honors and Awards:

Member WHO/FAO Board of Animal Mycoplasma Characterization. Participated in Board meeting in Paris, France on March 19 and 20, 1971.

Member, International Subcommittee on Taxonomy of the Mycoplasmatales.

Vice Chairman (1970-71) Mycoplasmology Section, American Society for Microbiology.

Project Officer, NIAID Research Contract PH 43-67-682, University of Aarhus, Denmark.

Secretary, NIAID Assembly of Scientists.

Participated in and jointly convened two Round Tables on mycoplasmas at the 21st annual meeting of the American Society for Microbiology, Minneapolis, Minnesota May 2-7, 1971.

Invited participant at a symposium on Mycoplasma Diseases of Plants and Insects at the annual meeting of the Entomological Society of America, Miami Beach, Florida November 30 - December 2, 1970.

Publications:

1. Tully, J. G. and Razin, S.: Acholeplasma axanthum sp. nov.: A new sterol nonrequiring member of the Mycoplasmatales. J. Bact. 103: 751-754, 1970.

2. Theodore, T. S., Tully, J. G. and Cole, R. M.: Polyacrylamide gel identification of bacterial L-forms and Mycoplasma species of human origin. Applied Microbiol. 21: 272-277, 1971.

3. Armstrong, D., Morton, V., Yu, B., Friedman, M. H., Steger, L., and Tully, J. G.: Pneumonias in dogs associated with Mycoplasma infections. Infect. and Immunity (in press).

4. Tully, J. G.: Mycoplasmas of laboratory animals. In Standardized Methods for Veterinary Microbiology. National Academy of Sciences-National Research Council, Washington, D. C. (in Press).

Serial No. NIAID-152
1. Microbiology
2. Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Antibody Production and Mechanisms of Hypersensitivity.

Previous Serial Number: Same

Principal Investigator: Dr. Sanford H. Stone

Other Investigators: Mr. Julius H. Goode, Jr.

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	-	-	24/12
Professional	24/12	-	-	24/12
Other	-	-	-	-

Project Description:

Project Subtitle I: Genetics of immune responses of guinea pigs.

Objectives:

To elucidate the mechanisms underlying immune responses of highly inbred guinea pigs to specific determinants on synthetic antigens and on microbial antigens and to study the genetic control of such responses.

Methods Employed:

Young and mature, male and female, Strain 2 and Strain 13 guinea pigs and their F_1 hybrids were sensitized to dinitrophenol-polylysine (DNP-PLL) and certain natural antigens and tested for cellular and humoral immune responses *in vivo* and *in vitro*.

Major Findings:

The control of immunization with DNP-PLL may not be specific and might instead reflect a general deficit in response to low doses of a wide selection of unrelated antigens, since sensitization with several proteins reveals a similar split (Strain 2 "responders" and Strain 13 "non-responders"). When high doses of antigen are injected, this difference disappears.

Significance to Bio-Medical Research and the Program of the Institute:

Genetic control of specific immune responses evidently involves complex mechanisms calling on multiple genes for the response. The simplistic view of an immune response obeying the yes-or-no, all-or-none control of a single gene is to be avoided.

Proposed Course of Project:

High and low dose responses should be examined to a whole spectrum of synthetic and bacterial antigens to see if a pattern of general deficiencies in immune response would yield information for optimal immunization schedules in animals and man as well as an insight into genetic control of immune responses.

Project Subtitle II: Genetics and pathogenesis of acute and chronic autoimmune encephalomyelitis.

Objectives:

To elucidate the mechanisms underlying resistance to autoimmune encephalomyelitis linked to age, sex or strain differences, in search for information on the etiology of the disease, or of demyelinating diseases in general.

Methods Employed:

Young and mature, male and female, Strain 2 and Strain 13 guinea pigs were sensitized with homologous spinal cord incorporated into Freund's complete adjuvant containing killed Mycobacterium tuberculosis. Various immunosuppressive techniques were applied: injection of cyclophosphamide, surgical removal of the depot of antigen-adjuvant emulsion, and immune deviation.

Major Findings:

In groups of guinea pigs sensitized with guinea pig spinal cord in Freund's complete adjuvant and later subjected to excision of the sites of antigen-adjuvant deposition, there were occasional animals showing remissions and relapses. A few guinea pigs with this type of chronic syndrome were in groups of animals pretreated with the spinal cord antigen in incomplete Freund adjuvant prior to sensitization (immune deviation).

Significance to Bio-Medical Research and the Program of the Institute:

It is still debatable whether viral, allergic or autoimmune mechanisms or combinations of these, are responsible for human demyelinating disease. Good animal models must be devised to clarify these problems and to serve in therapeutic testing for demyelinating diseases and autoimmune diseases in general. An experimental disease showing remissions and relapses would be more reminiscent of multiple sclerosis than the usual acute autoimmune encephalomyelitis seen in guinea pigs.

Proposed Course of Project:

To study the heterogeneity of types of autoimmune encephalomyelitis in relation to the heterogeneity of the immune responses to the myelin antigen, and in relation to the heterogeneity of forms of human encephalomyelitis (post-vaccinal, post-infectious, autoimmune and idiopathic).

Project Subtitle III: Studies on cellular manifestations of hypersensitivity and immunity in guinea pigs: transplantation of immunologically competent tissues.

Objectives:

Correlation of the cellular developments in lymphoid tissues draining the sites of injection of antigens with the capacity of the nodes to transfer either hypersensitivity or antibody producing capacity.

Methods Employed:

Inbred guinea pigs were sensitized with various antigens incorporated into complete or incomplete Freund's adjuvant. Lymph node cells, peritoneal exudates and circulating leukocytes were transferred at various times after sensitization into inbred and random-bred recipients. Recipients were observed for skin reactivity, tested for circulating 19S, $7S\gamma 1$, and $7S\gamma 2$ antibodies, and tissues examined for evidence of autoimmune disease.

Major Findings:

Prior injection of spinal cord in incomplete Freund's adjuvant will protect guinea pigs against the autoimmune encephalomyelitis enduced by injection of spinal cord in complete Freund's adjuvant. This protection prevents the acute disease and delays onset of a chronic disease (see Subtitle II). The mechanism of this type of immune deviation was explored by attempts to induce immune deviation with protein antigens in incomplete Freund's adjuvant and to transfer the reduced capacity with lymphoid cells. When protein in incomplete adjuvant is injected prior to injection of the same antigen in complete adjuvant, the reactivity in the guinea pig is deviated toward Jones-Mote (cutaneous basophil hypersensitivity) rather than the classic delayed response.

Significance to Bio-Medical Research and the Program of the Institute:

The types of cells required for transfer of adoptive immunity are of interest for the reconstruction of the immune response at the cellular level. The adoptive transfer is a powerful tool for study of the cell-cell interactions newly recognized of great importance in immune responses.

Proposed Course of Project:

Transfers involving cell suspensions of known composition (from different sources or gradient preparations from such sources) and of slices of lymphoid tissues will be undertaken to assess the requirements for cell-to-cell association or of certain tissue architecture for maximal immune response.

Honors and Awards:

Associate Editor, Journal of Immunology

Professorial Lecturer, Howard University, Washington, D. C. (immunology)

Representative for the American Association of Immunologists on the Advisory Committee, Office of Biomedical Studies, FASEB.

Publications:

Davey, M. Jean, Asherson, G. L., and Stone, S. H.: Selective and specific inhibition of 24 hour skin reactions in the guinea pig. III. Depression of cytophilic and haemolytic antibodies by pretreatment with antigen and the effect of irradiation. Immunology 20:513- 1971 (in press)

LABORATORY OF BIOLOGY OF VIRUSES

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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
July 1, 1970 through June 30, 1971

The optimism shared by the members of this laboratory when we occupied our newly renovated laboratory space in January 1970 was clearly justified. Unification of the laboratory in Building 5 has provided a strong, positive working environment. It has resulted in greater interaction between members of the laboratory and more effective utilization of equipment and other resources. The curtailment on hiring represents the most important factor that is restricting the effective operation of the laboratory. A number of ongoing programs reported in last year's annual report that were providing new information have not been continued and are not reported in this year's report. These programs were discontinued when visiting scientists left the laboratory and they could not be replaced.

A major change this past year was the reorganization of the Laboratory of Biology of Viruses. A particular source of gratification has been the recognition given to Drs. Martin, Moss and Rose, each of whom have developed important new areas of investigation and were appointed section heads. In addition, Dr. Martin presented his research findings at the Cold Spring Harbor Annual Symposium and he has been asked to give the Tumor Virus Course at Cold Spring Harbor during the summer of 1971. Dr. Moss has organized a section on rifampicin derivatives at the 2nd International Congress for Virology and has been asked to chair the session. Dr. Salzman has been reappointed an editor of the Journal of Virology for an additional four years. He has also become a member of the Virology Study Section for a four-year period.

As detailed in the individual project reports, members of the laboratory have given a number of talks at universities and at various meetings.

The general area of research of this laboratory continues to be molecular virology. Members of the Laboratory of Biology of Viruses have used knowledge of molecular biology in order to gain an understanding as to how human and animal viruses affect cells and how they replicate. Studies on the mechanism of transformation of cells by oncogenic viruses continue as a major area of investigation. This past year has been successful and during this period many notable findings have been made.

PRINCIPAL RESEARCH FINDINGS

Proteins Present In Metaphase Chromosomes Have Been Determined

Three major groups of chromosomal proteins have been selectively extracted from Chinese hamster metaphase chromosomes. These are the non-histone acid soluble proteins (ca. 20 proteins), the histones (5 major proteins) and the acid-insoluble proteins (ca. 45 proteins). Acid insoluble proteins range in molecular weight from 15,000 to 180,000 daltons. Many of the non-histone proteins have been found to be glycoproteins as determined by their incorporation of ^{3}H-glucosamine. By comparing the number and types of proteins in chromosomes with those in other nucleoproteins, metaphase chromosomes are found to be enriched in various non-histone acid soluble species and acid insoluble species. Now that all Chinese hamster chromosomal proteins have been catalogued and partially characterized it should be possible to determine the unique location or distribution of the various proteins in chromosome size groups.

The histones contained in Chinese hamster metaphase chromosomes when examined by polyacrylamide gel electrophoresis are identical in type and relative amounts as compared with histones in interphase chromatin of Chinese hamster cells. Chinese hamster metaphase chromosomes were separated into three size groups, and a comparison of the histones revealed they were the same for each chromosome class.

Preliminary studies on the synthesis of histones in this system indicate that these proteins are synthesized predominantly during the S phase, concomitant with DNA synthesis, although a low level of synthesis occurs throughout the cell cycle. The f2a2-f2b histone and the f2a1 histone are synthesized in a coordinate manner and in a fixed proportion. This suggests that they may be transcribed from a unique polycistronic messenger RNA. Differences in time and rate of synthesis of the various histones contained in different size chromosomes are not apparent. (Lake, Goidl, Salzman)

The Structure Of The SV-40 DNA Replicative Intermediate Has Been Defined

The structure of replicating SV40 DNA has been established by electron microscopy and biochemical analysis. Newly replicated DNA is not covalently linked to the parental SV40 DNA template. This finding excludes one current model proposed for replication of covalently closed circular DNA, the rolling circle model. In addition, there is no covalent link between the two newly replicated DNA strands. Eighty to ninety percent of the isolated replicating molecules contain superhelical regions which show that a swivel is absent in the replicating molecule. Replicating molecules with these unique features have not been described previously. It has been possible to effect a fractionation of these molecules based on the extent to which they have replicated. The newly replicated DNA can then be isolated from the replicating molecules by alkaline sedimentation. By this procedure, SV40 DNA sequences which correspond to limited regions of the SV40 genome have been obtained. These fragments are of great value in locating various gene functions within the viral genome. (Sebring, Thoren, Salzman, LBV; Kelly, LVD)

Factors That Control SV40 DNA Expression Have Been Examined

A restriction enzyme isolated from E. coli B will cleave SV40 DNA Comp. I at a single site. The product of this cleavage is a linear 14S SV40 DNA molecule (SV40, Comp. III). The infectivity of this linear DNA has been examined, as has its ability to induce T-antigen synthesis and the synthesis of viral coat proteins (V antigen). Comp. III has only 1/10 the infectivity of Comps. I and II. However, it is as effective as Comps. I and II in inducing the synthesis of T and V antigens. The finding of reduced infectivity combined with the ability to effectively stimulate V-antigen synthesis poses some interesting new questions. It is possible that transcriptional and translational controls which normally couple the synthesis of V antigen to viral DNA synthesis are abolished when a linear rather than a circular DNA molecule is used to initiate an infectious cycle. (Steimatsky, Sebring, Salzman)

The Site Of Integration Of SV40 DNA In Transformed Cells Has Been Examined

In one SV40 transformed cell line, the site of integration of the viral genome fractionated with the intermediate sized chromosomes. Since this cell line contained low levels of infectious virus, the full significance of this finding is not clear. In a second transformed line in which no infectious virus was present, the viral genome was distributed randomly. However, this cell line was characterized by interphase nuclei that were easily ruptured, and this is likely to have affected the results. The chromosome isolation techniques have been further modified and a number of new transformed cell lines are being reexamined. (Patch, Salzman, LBV; Levine, NCI)

SV40 Gene Products Can Be Modulated At The Post-Transcriptional Level

SV40 specific RNA present in the nuclei of cells transformed by SV40 or acutely infected with SV40 had sedimentation values exceeding that complementary to a single strand of SV40 DNA. Whether these large RNA molecules contain several copies of SV40 genetic information and/or interspersed mammalian polynucleotide sequences is currently under study.

The finding of high molecular weight RNA containing SV40 polynucleotide sequences in the nuclei of transformed cells prompted an examination of the processing of these molecules during their migration into the cytoplasm in mouse cells acutely infected with SV40 and in established virus transformed cell lines. By 8 hours, nuclei of infected cells contain both "early" and "late" species of virus-specific RNA. The cytoplasm, however, only contains "early" SV40 RNA. Similar results were obtained late in transforming infection as well as in established SV40 transformed lines. These findings suggest that post-transcriptional modulation of viral gene products exists as a control mechanism in animal cells. (Martin, Byrne)

One Or Two Copies Of SV40 DNA Are Present In Established, Virus-free, Transformed Cell Lines

In five out of six virus-free SV40 transformed lines examined, an average of one SV40 genome equivalent per cell was detected; three SV40 DNA equivalents were found in the sixth viral transformed line. 0.45 and 0.50 SV40 DNA copies were measured in normal mouse and African green monkey cells, respectively. (Gelb, Martin)

The presence of SV40 genetic material in "abortively transformed" mouse cells has also been studied. These lines were infected with a high multiplicity of SV40 and originally selected in factor-free medium. They are T antigen negative, appear to be contact inhibited ("flat"), and do not yield virus following cell fusion. Of the three abortively transformed lines examined, two contained about five copies of SV40 DNA per cell while the third was indistinguishable from normal mouse DNA. Two subclones of one of the abortively transformed lines contained three and ten copies, respectively. There was no indication that the SV40 genome detected in these "flat" lines was incomplete. (Gelb, Martin, Byrne, LBV; Smith, Bionetics Research Labs., Bethesda, Md.)

DNA Copies Of RNA Tumor Virus Genetic Information Have Been Detected In Normal And Virus Transformed Animal Cells

The *in vitro* double-stranded DNA fragments generated by the RNA dependent DNA polymerase associated with KiMuLV represents 40-60% of the newly synthesized DNA. This double-stranded DNA consists of at least two components: the major components, containing about 80% of the double-stranded product, has an informational content equivalent to 25% of the viral genome; the remaining DNA reanneals at a rate which suggests that it represents the entire RNA viral genome. When labeled double-stranded DNA was used to probe normal and viral transformed cells, 8 to 50 copies of viral genetic material were detected in both. In no case were normal cells clearly distinguishable from cells transformed by murine leukemia viruses. Similar studies were carried out with labeled single-stranded *in vitro* DNA. Although the H^3 single-stranded DNA probe reacted with normal mouse DNA, a significantly larger fraction formed stable duplex molecules when incubated with KiMuLV transformed cell DNA. Experiments are currently in progress to determine whether the entire viral genome or a portion is integrated into the transformed cell chromosome. (Martin, Gelb, Byrne, LBV; Aaronson, NCI)

Specific SV40 Fragments Can Be Obtained By Nuclease Treatment

SV40 DNA circles are cleaved across both strands in the same local region by the restriction enzymes from either *E. coli* B or *E. coli* K lysogenized by P1 phage. Neither enzyme produces a significant amount of acid soluble DNA products after prolonged incubation. When the linear DNA produced by the coli restriction enzyme is treated with exonuclease III and the single strands of the DNA are tested, the limit product has about one-half of the original length. Thus, no large duplex piece of SV40 DNA remains resistant to attack by exonuclease III after the initial cleavage by the coli restriction enzymes.

The restriction enzyme from H. Influenzae produces smaller pieces of SV40 DNA than the coli enzymes. In addition, this enzyme cleaves all SV40 DNA I circles while digestion with the B and P1 enzymes always shows a small surviving fraction of resistant circles. Sucrose gradient sedimentation indicates three size classes of single-stranded DNA are produced by the Hemophilus enzyme and as with the B and P1 enzymes no extremely small pieces are produced. (DeFilippes)

Plaque Mutants Of SV40 Differ In Their Coat, Protein Structure

SV40 virus particles contained a minimum of four polypeptide components whose molecular weights range from 45,000 to 16,000 daltons. The major structural component was the capsid protein consisting of two subunits. Comparison of a large plaque morphology SV40 mutant with a small plaque virus strain by double-isotope labeling revealed a generally similar polypeptide pattern by polyacrylamide gel electrophoresis.

Employing the technique of electrofocusing, which involves separation of proteins of different isoelectric points by electrophoresis in a pH gradient, a distinct difference between the capsid proteins of the SV40 mutants could be demonstrated. By this technique, the other protein components of these mutants were found to be very similar. Isoelectric focusing of a mixture of capsid proteins from both large and small plaque SV40 mutants labeled with different isotopes revealed a charge difference of less than 0.10 pH unit. This result indicates a difference exists in either amino acid composition or protein conformation of the capsid proteins of these plaque morphology mutants. (Barban)

Early Stages Of Vaccinia Virus Assembly Can Be Synchronized By The Use Of Rifampicin

Assembly of immature vaccinia particles was interrupted in HeLa cells treated with rifampicin despite the synthesis of all resolvable structural polypeptides. The primary action of rifampicin appeared to occur during the stage of envelope formation. It was demonstrated that the trilaminar membranes of irregular contour which accumulate in the presence of rifampicin are precursors of virus envelopes. When rifampicin was removed under controlled conditions, synchronous transitions were observed as the precursor membranes rapidly converted into uniformly curved envelope units with a 10- to 12-nm coat on the convex surface. This process did not require new RNA or protein synthesis. Nucleation of immature particles was the first evidence of core differentiation and began within 5 to 10 minutes. Structurally mature virions were present by 1-2 hours and coincided with an increase in the late particulate RNA polymerase activity. High resolution autoradiography was used to study the stage at which viral deoxyribonucleic acid was incorporated into the virus particles. (Moss, Garon, Rosenblum, LBV; Grimley, NCI)

Rifampicin-resistant Vaccinia Virus Mutants Complement The Growth of Drug-sensitive Virus

A rifampicin-resistant vaccinia virus mutant was isolated from wild-type virus stocks. Growth of the mutant was inhibited only slightly at concentrations of rifampicin which completely prevented the growth of drug-sensitive virus and at no concentration tested was there a detectable effect on viral envelope formation. Furthermore, the mutant was capable of rescuing wild-type virus during mixed infection in the presence of rifampicin. (Moss, Garon, Rosenblum, LBV; Grimley, NCI)

Cleavage Of A Precursor Of A Vaccinia Virus Structural Polypeptide Occurs During Virus Assembly And Can Be Prevented By Amino Acid Analogues

Polypeptide 4a, a major vaccinia structural polypeptide which was previously shown to form from a high-molecular-weight precursor is made after the period of viral DNA synthesis. Pulse-chase experiments demonstrated that a period of 1 to 2 hours is required for a 50% conversion of precursor to product. The kinetics of incorporation of structural polypeptides into virus particles and the presence of 4a exclusively in a particulate form at all times examined suggested that cleavage of the precursor occurs after, or immediately prior to, association with developing virus particles. Under conditions in which breakdown of polypeptide P4a was inhibited by adding rifampicin or amino acid analogues after the period of viral DNA synthesis, isolated virus particles contained significant amounts of this polypeptide localized within the virus core, which is also the site of 4a. (Moss, Garon, Rosenblum, LBV; Grimley, NCI)

Structure And Transcription Of Adenovirus-associated Virus (AAV) DNA

The AAV DNA strands which are more heavily substituted with bromodeoxyuridine band at a greater density in CsCl and would thus be expected to contain more thymidine. This was confirmed by direct measurement of the base composition of each strand species. The "heavy" strands contained 20% more thymidine than the "light" strands. Complementarity of the strand species was reflected by their A:T equivalence.

In vivo synthesized AAV-specific RNA (RNA which hybridizes with AAV DNA) has a base composition like that of the "light" DNA strands. This suggests that the "heavy" strands are the transcriptional template *in vivo*.

In vivo synthesized AAV-specific RNA hybridizes only with "heavy" strands which confirms the template function of this strand species. Tentatively, these strands can be designated "minus" strands, while the non-transcribed "light" strands are designated "plus" strands. (Rose, Carter, Koczot)

The Site And Extent Of Integration Of SV40 DNA In An Adenovirus SV40 Hybrid Has Been Measured

Intergeneric hybrids between adenovirus and SV40 genomes are useful models for studying the mechanism of DNA integration as well as the genetic determinants of viral oncogenesis. Electron microscopic mapping techniques can be used to analyze the detailed structure of hybrid DNA from these animal viruses. Based on electron microscopy, the structure of an Ad.7-SV40 hybrid genome ($E46^+$ PL1) can be reconstructed as follows: (1) the hybrid genome contains 16% less Ad.7 DNA than the non-hybrid Ad.7 genome, (2) this deletion consists of the segment of DNA mapping between 0.05 and 0.21 molecular lengths in the non-hybrid Ad.7 DNA molecule, (3) the deleted DNA has been partially replaced by an amount of heterologous DNA equivalent to 75% of the complete SV40 genome.

The representation of SV40 DNA sequences in an Ad.7-SV40 hybrid genome ($E46^+$ PL1) was estimated by competitive DNA/DNA hybridization. It was found that the hybrid genome contained 45-50% of sequences present in the complete SV40 DNA molecule. Coupled with mapping data this finding suggests the possibility that a repetitive segment of SV40 DNA sequences may be included in the SV40 DNA which is integrated into the Ad.7 genome. (Rose, Polmar, LBV; Kelly, LVD; Lewis, NINDB).

Purified Kilham Rat Virus (KRV) Contains A DNA Polymerase In The Virion

Purified KRV when dissociated by both electrophoresis in polyacrylamide gels and by agarose gel filtration contains 3 major protein subunits. These viral proteins have molecular weights of approximately 72,000, 62,000 and 55,000 daltons. The protein component with a molecular weight of 62,000 daltons comprised about 75.5% of the total virion protein and is believed to be the capsid protein. The other two protein components are likely to be internal proteins. DNA polymerase found associated with purified KRV prefers native, double-stranded DNA as a template. Maximal activity occurs at pH 9.0 and 0.003 M $MgCl_2$. The enzyme is inhibited by EDTA, KCl and p-chloromercuribenzoate. All four deoxynucleotide triphosphates are required for maximal activity. The requirements for a native DNA template and inhibition by p-chloromercuribenzoate are properties that distinguish KRV-DNA polymerase from the DNA polymerase isolated and purified by Kornberg.

KRV-DNA extracted by both phenol and 2% SDS is a linear single-stranded molecule. The linear configuration of the DNA is also supported by the fact that it is hydrolysed by exonuclease I. As measured in the electron microscope, the DNA is 1.5 ± 0.206 μ in length. This is the first documented report of a linear single-stranded DNA molecule. (Lois Salzman, White, LBV; Kakefuda, NCI)

Serial No. NIAID-80A
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical characterization of nucleic acids of transformed cells

Previous Serial No.: Same

Principal Investigators: Dr. Malcolm Martin
Dr. Lawrence Gelb

Other Investigators: Mrs. Janet Byrne

Cooperating Units: Dr. Kenneth Takemoto, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases

Dr. Stuart Aaronson, Viral Carcinogenesis Branch, National Cancer Institute

Dr. Helene Smith, Bionetics Research Laboratories, Bethesda, Md. 20014

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	66/12			66/12
Professional:	24/12			24/12
Other:	42/12			42/12

Project Description:

Objectives:

To elucidate the process of viral DNA transcription under conditions of productive and transforming infection.

To quantitate the amount of SV40 genetic material integrated into the chromosomes of normal and viral transformed mammalian cells.

To examine the site(s) of insertion of oncogenic viral DNA in the animal cell genome; to determine the kinetics of SV40 DNA integration into the mammalian cell chromosome.

To detect and characterize RNA tumor virus genetic material in normal and transformed animal cells using labeled in vitro DNA generated with the RNA dependent DNA polymerase associated with these agents.

Methods Employed:

SV40 DNA Transcription. Nuclear and cytoplasmic RNA's were prepared from cells acutely infected with SV40 and from established viral transformed cell lines. SV40 specific RNA was detected by standard DNA-RNA hybridization techniques. The size distribution of viral specific RNA was analyzed by rate zonal centrifugation using 15-30% sucrose gradients.

SV40 Integration in Mammalian Cells. The number of SV40 genome equivalents in several cell lines was determined from the reassociation kinetics of SV40 DNA using hydroxyapatite columns. Cellular DNA, prepared from virus-transformed lines, was also separated into unique and repetitive fractions by passage through hydroxyapatite following mechanical shear, heat denaturation and reassociation. The number of SV40 genome equivalents was then redetermined in each fraction. The kinetics of viral DNA integration were studied by separating high molecular weight SV40 transformed cellular DNA from viral DNA by passage through sucrose in a B-IV zonal rotor. The high molecular weight cellular DNA so obtained was then analyzed for SV40 sequences on hydroxyapatite.

Identification of RNA Tumor Virus Genetic Material in Animal Cells. In vitro DNA was prepared from Kirsten murine leukemia virus (Ki-MuLV) by incubating detergent disrupted virus preparations with tritiated deoxynucleotide triphosphates. Such reaction mixtures were phenol extracted, exposed to pancreatic ribonuclease in low salt, and passed through hydroxyapatite columns to separate single-stranded (ss) from double-stranded (ds) DNA products. The informational content of the ds DNA generated in the in vitro reaction was determined by analysis of reassociation kinetics. In addition, both the ss and ds DNA products were used as a probe to quantitate RNA tumor virus genetic material in normal and transformed cells.

Major Findings:

SV40 DNA Transcription. The size distribution of SV40 specific RNA present in the nuclei of cells transformed by or acutely infected with SV40 was examined. In both cases RNA with sedimentation values exceeding that complementary to a single strand of SV40 DNA could be demonstrated. Experiments are still in progress to determine whether these large RNA molecules contain several copies of SV40 genetic information and/or interspersed mammalian polynucleotide sequences.

The finding of high molecular weight RNA containing SV40 polynucleotide sequences in the nuclei of transformed cells prompted us to examine the processing of these molecules during their migration into the cytoplasm. Our studies indicate that by 8 hrs., the nuclei of infected mouse cells contain both "early" and "late" species of virus-specific RNA. The cytoplasm, however, only contains "early" SV40 RNA. Similar results were obtained late in transforming infection as well as in established SV40 transformed lines. This finding of post-transcriptional control of SV40 gene products was suggested by experiments described in last year's annual report.

SV40 Integration in Mammalian Cells. In five out of six virus-free SV40 transformed lines examined, an average of one SV40 genome equivalent per cell was detected; three SV40 DNA equivalents were found in the sixth viral transformed line. 0.45 and 0.50 SV40 DNA copies were measured in normal mouse and African green monkey cells, respectively. Preliminary experiments performed with fractionated transformed cellular DNA suggests that the integrated viral genetic material is more closely associated with the repetitive host sequences in the single line examined.

In another series of experiments carried out in collaboration with Dr. Helene Smith, we examined the presence of SV40 genetic material in "abortively transformed" mouse cells. These lines, which were infected with a high multiplicity of SV40 and originally selected in factor-free medium, are T antigen negative, appear to be contact inhibited ("flat") and do not yield virus following cell fusion. Of the three abortively transformed lines examined, two contained about five copies of SV40 DNA per cell while the third was indistinguishable from normal mouse DNA. Two subclones of one of the abortively transformed lines contained three and ten copies, respectively. There was no indication that the SV40 genome detected in these "flat" lines was incomplete.

Identification of RNA Tumor Virus Genetic Material in Animal Cells. The *in vitro* ds DNA fragments generated by the RNA dependent DNA polymerase associated with KiMuLV represents 40-60% of the newly synthesized DNA. This ds DNA consists of at least two components: the major component, containing about 80% of the ds product, has an informational content equivalent to 25% of the viral genome; the remaining DNA reanneals at a rate which suggests that it represents the entire RNA viral genome. When labeled ds DNA was used to probe normal and viral transformed cells, 8 to 50 copies of viral genetic material was detected in *both*. In no case were normal cells clearly distinguishable from cells transformed by murine leukemia viruses. Similar studies performed with labeled ss *in vitro* DNA differentiated normal from transformed cell lines but considerable complementarity was observed in both. It is not clear at present whether only a portion or the entire viral genome is present in cells transformed by these oncogenic RNA viruses.

Significance to Biomedical Research and the Program of the Institute:

One of our goals is to delineate those viral genes necessary to induce and/or maintain the transformed state. Since the processing of SV40-specific RNA may be different in cells that are destined to be transformed as opposed to those that support productive infection, a more thorough understanding of the control mechanisms involved may enable one to regulate the type of cellular response following exposure to these agents.

The ultimate goal of this project is to understand the integration of viral genetic material into the mammalian chromosome and the relationship of this process to oncogenesis. The determination of the time course of integration, the number of copies of viral DNA present in cells undergoing transforming infection and the nature of the integration site in the animal cell genome may permit one to separate the various steps involved and possibly interrupt these molecular events with appropriate chemotherapeutic agents.

Proposed Course of the Project:

Emphasis will be placed on understanding the mechanisms of integration of both RNA and DNA tumor virus genetic material into the host chromosome. Mammalian DNA from cells undergoing both productive and transforming infection will be examined to determine when stable integration occurs. Fractionated transformed cell DNA will be analyzed to ascertain the nature of the host polynucleotide sequences adjacent to the integration site. Further investigation of the seemingly functionless SV40 DNA in abortively transformed cells will be carried out. We shall also study the nature of the genetic information shared by RNA tumor viruses and normal cells.

Further studies to characterize the nature of the "post-transcriptional" control of SV40 gene products in mouse cell will be carried out.

Honors and Awards:

Invited lectures at:

1. Princeton University, Princeton, New Jersey
2. Institute for Cancer Research, Philadelphia, Pennsylvania
3. Harvard University, Cambridge, Massachusetts
4. New York State Department of Health, Albany, New York

Publications:

Martin, M. A. and Byrne, J. C.: Sedimentation properties of SV-40 specific RNA present in green monkey cells during productive and abortive infection. J. Virol. 6: 463-469, 1970.

Aaronson, S. A. and Martin, M. A.: Transformation of human cells with different forms of SV-40 DNA. Virology 42: 848-856, 1970.

Takemoto, K. K. and Martin, M. A.: SV-40 thermosensitive mutant: Synthesis of viral DNA and virus-induced proteins at non-permissive temperature. Virology 42: 938-945, 1970.

Martin, M. A.: Characteristics of SV-40 DNA transcription during lytic infection, abortive infection and in transformed mouse cells. Cold Spring Harbor Symposium on Quantitative Biology 35: 833-841, 1970.

Serial No. NIAID-80G
1. Biology of Viruses
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Viral Protein Synthesis and Assembly of Virus Particles

Previous Serial Number: Same

Principal Investigators: Dr. Bernard Moss
Dr. Claude F. Garon
Miss Edith N. Rosenblum

Cooperating Units: Dr. Philip M. Grimley, Laboratory of Pathology, NCI, Project NCI 555A

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	42/12		12/12	54/12
Professional:	24/12		12/12	36/12
Other:	18/12			18/12

Project Description:

Objectives:

1. Determine the regulatory mechanisms involved in sequential synthesis of viral proteins.

2. Elucidate the mechanisms involved in the assembly of viral particles.

3. Determine the mode of action of anti-viral drugs.

Methods Employed:

Vaccinia virus was grown in HeLa cells and purified by density gradient sedimentation. DNA, RNA and protein synthesis in infected cells was measured by the incorporation of radioactive precursors and the separation of macromolecules by gel filtration, gel electrophoresis, column chromatography and zonal centrifugation. Specific viral proteins were further identified by immunological methods. Electron microscopy and radioautography was used to study virus morphogenesis.

Major Findings:

Early stages of vaccinia virus assembly can be synchronized by the use of rifampicin.

Assembly of vaccinia virus envelopes and immature vaccinia particles was interrupted in HeLa cells treated with rifampicin. The primary action of rifampicin on vaccinia morphogenesis appeared to occur during the stage of envelope formation. It was demonstrated that the trilaminar membranes of irregular contour which accumulate in the presence of rifampicin are precursors of virus envelopes. When rifampicin was removed under controlled conditions, synchronous transitions were observed as the precursor membranes rapidly converted into uniformly curved envelope units with a 10- to 12-nm coat on the convex surface. These experiments provided an opportunity to examine the sequence of some early events in vaccinia morphogenesis. Initially, nascent envelopes remained in clusters around dense viroplasm. Large numbers of single immature particles appeared within 10 min. Nucleation of immature particles was the first evidence of core differentiation and began within 5 to 10 min. Development and structurally mature virions were present by 2 hr after the removal of rifampicin. An appreciable increase in the late particulate RNA polymerase activity could not be demonstrated until after the rifampicin effect on the formation of the viral envelope was completely reversed and mature particles were evident.

High resolution autoradiography showed that viral deoxyribonucleic acid, which labeled with ^{3}H-thymidine during rifampicin treatment, was incorporated by the mature vaccinia which formed after rifampicin was removed. Concentration of the viral deoxyribonulceic acid in core material evidently occurred after envelope assembly, probably coincident with nucleoid formation. Cytoplasmic crystalloid bodies accumulated during rifampicin treatment; they appeared morphologically identical to vaccinia nucleoids and were heavily labeled by ^{3}H-thymidine.

Vaccinia virus particles can be assembled from polypeptides made in the presence of rifampicin.

Vaccinia viral polypeptides, labeled in the presence of rifampicin, were incorporated into virus particles after rifampicin was removed. All structural polypeptides, resolved by polyacrylamide gel electrophoresis, were labeled in the presence of rifampicin. The specific effect of rifampicin on the formation of the vaccinia viral envelope was reversed within 10 min even in the presence of NaF or inhibitors of protein or nucleic acid synthesis. Further maturation of some virus particles, which were characterized by their ultrastructure and isolated by sucrose gradient sedimentation, proceeded in the presence of inhibitors of protein synthesis. In contrast, addition of NaF or actinomycin D almost completely blocked the development of mature virus particles. Rifampicin acted specifically at the stage of envelope formation and did not prevent later steps in maturation.

Rifampicin-resistant vaccinia virus mutants complement the growth of drug-sensitive virus.

A rifampicin-resistant vaccinia virus mutant was isolated from wild-type virus stocks. The mutant was purified by sucrose density gradient centrifugation and had an infectivity, electron microscopic appearance, and polypeptide composition that was similar to the wild-type virus. Growth of the mutant was inhibited only slightly at concentrations of rifampicin which completely prevented the growth of drug-sensitive virus and at no concentration tested was there a detectable effect on viral envelope formation. The mutant virus was capable of rescuing wild-type during mixed infection in the presence of rifampicin; after simultaneous infection the ratio of genomes in the progeny reflected that of the original inoculum. Superinfection experiments demonstrated that heat inactivated mutant was capable of efficiently rescuing wild-type virus and that extensive replication of the mutant genome was not required for this. Characteristic rifampicin blocked viral forms, more mature forms and intermediate structures were seen in the same ultrathin cell sections after simultaneous infection with low multiplicities of mutant and wild-type virus. In cells simultaneously infected with high multiplicities of mutant and wild-type virus, rifampicin had no detectable effect on viral envelope formation. No difference was found in pulse-labeled polypeptides synthesized in rifampicin-treated cells infected with either mutant virus, wild-type virus or both when analyzed by SDS-polyacrylamide gel electrophoresis. After a 4 hour chase, cleavage of the high molecular weight precursors of structural polypeptides was inhibited in cells infected with wild-type virus but occurred to similar extents in cells infected with mutant alone and cells simultaneously infected with mutant and wild-type virus.

Cleavage of a precursor of a vaccinia virus structural polypeptide occurs during virus assembly and can be prevented by amino acid analogues.

Polypeptide 4a, a major vaccinia structural polypeptide which was previously shown to form from a high-molecular-weight precursor is made after the period of viral deoxyribonucleic acid (DNA) synthesis. Pulse-chase experiments demonstrated that a period of 1 to 2 hr is required for a 50% conversion of precursor to product. The rates of incorporation of polypeptides into virus particles were examined. The kinetics of incorporation of labeled 4a and other major structural polypeptides into virus particles were similar, despite the additional time required for the formation of 4a from its precursor. Furthermore, 4a was present exclusively in a particulate form at all times examined. Both observations suggested that cleavage of the precursor occurs after, or immediately prior to, association with developing virus particles. Polypeptide P4a was previously identified as the probable precursor of 4a and and is not ordinarily found in detectable amounts in virus particles. Under conditions in which breakdown of P4a was inhibited by adding rifampin or amino acid analogues after the period of viral DNA synthesis, isolated virus particles contained significant amounts of this polypeptide. Further analysis showed that P4a was localized within the virus core, which is also the site of 4a. Synchronization of virus assembly after the removal of rifampin was shown to be useful for studying the integration of polypeptides into a particulate fraction of the cytoplasm.

Significance to Biomedical Research and the Program of the Institute:

Detailed knowledge of the way viruses dominate the metabolic activities of their host cells and regulate their own biosynthetic activities may provide insights for rational chemotherapy. The antibiotic rifampicin has been shown to act at a specific stage in virus assembly. Derivatives of this drug may prove to have therapeutic value. Rifampicin has already proved valuable in studying virus assembly, a process that is poorly understood but which is potentially a specific target for anti-viral drugs.

Proposed Course of the Project:

Emphasis will be placed on the related problems of the assembly of vaccinia virus particles and the mechanism of action of rifampicin. The effectiveness of certain other rifamycin derivatives will be tested. Additional rifampicin-resistant mutants will be isolated and intensively studied to find the basis for drug resistance. Glycoprotein synthesis in vaccinia virus infected cells will be studied and characterization of vaccinia virus structural proteins and their precursors will be continued.

Honors and Awards:

Chairman of session on "Rifampicin and Related New Antiviral Agents" at Second International Congress of Virology held in Budapest, Hungary during June, 1971.
Leader of tutorial seminar on "Biochemistry of Antiviral Substances", fall term, 1970.

Invited lectures at:

University of Connecticut Health Center
Massachusetts Institute of Technology
Johns Hopkins University School of Medicine

Served as guest on graduate committee of George Washington University.

Publications:

Katz, E., Grimley, P. M. and Moss, B.: Reversal of antiviral effects of rifampicin. Nature 227: 1050-1051, 1970.

Grimley, P. M., Rosenblum, E. N., Mims, S. J. and Moss, B.: Interruption by rifampicin of an early stage in vaccinia virus morphogenesis: Accumulation of membranes which are precursors of virus envelopes. J. Virol. 6, 519-533, 1970.

Katz, E. and Moss, B.: Formation of a vaccinia virus structural polypeptide from a higher molecular weight precursor: Inhibition by rifampicin. Proc. Natl. Acad. Sci. 66: 677-684, 1970.

Katz, E. and Moss, B.: Vaccinia virus structural polypeptide derived from a high-molecular-weight precursor: Formation and integration into virus particles. J. Virol. 6: 717-726, 1970.

Moss, B., Rosenblum, E. N. and Grimley, P. M.: Assembly of vaccinia virus particles from polypeptides made in the presence of rifampicin. Virology, in press.

Moss, B., Rosenblum, E. N. and Grimley, P. M.: Assembly of virus particles during mixed infection with wild-type vaccinia and a rifampicin-resistant mutant. Virology, in press.

Serial No. NIAID-80H
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Characterization and Synthesis of Kilham Rat Virus.

Previous Serial No.: Same

Principal Investigator: Dr. Lois Ann Salzman

Other Investigators: Mr. Wesley L. White

Cooperating Units: Dr. T. Kakefuda, National Cancer Institute

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	36/12			36/12
Professional:	12/12			12/12
Other:	24/12			24/12

Project Description:

Objectives:

To study the protein content of purified Kilham rat virus and to determine the molecular weight of the protein components.

To determine if the purified virion contains enzymatic activity and to describe the properties of any virion associated enzymes.

To characterize the DNA from the virion by determining its size and configuration.

Methods Employed:

KRV proteins were labeled with radioisotope by the addition of mixed ^{3}H-L amino acids and then purified by at least two isopycnic centrifugations in cesium chloride. The virus suspension was disrupted in 2% sodium dodecyl sulfate (SDS) and 1% β mercaptoethanol and then layered on a 7.5% SDS-polyacrylamide gel. The gels were electrophoresed for 16 hours at 4 mamps per gel and then sliced. The slices were counted for radioisotope content. Proteins of known molecular weight were added to some gels as standards along with the disrupted virions. These gels were stained with Coomassie blue and the standard protein bands used to determine the molecular weight of the virion proteins. The KRV-protein subunits were also separated by agarose gel filtration in 6M guanidine-HCl.

KRV virions purified by two cesium chloride equilibrium centrifugations and in some cases by an additional velocity sedimentation in sucrose were tested for DNA polymerase activity. The pH optimum, concentration of enzyme, length of incubation, template specificity, salt and nucleotide requirements of the reaction were determined. Several compounds reported to be inhibitory to other DNA polymerases were used to determine their effect on the KRV-DNA polymerase. The kinetics of the reaction was also studied.

The DNA was extracted from purified KRV by both phenol extraction and by 2% SDS at 50° C. KRV-DNA extracted by both procedures was observed in an electron microscope using cytochrome C or DEAE dextran monolayer. The DNA was also exposed to exonuclease I, an enzyme which hydrolyses specifically linear, single-stranded DNA.

Major Findings:

Purified KRV when dissociated by both electrophoresis on polyacrylamide gels and by agarose gel filtration contains 3 major protein subunits. These viral proteins have molecular weights of approximately 72,000, 62,000 and 55,000 daltons. The protein component with a molecular weight of 62,000 daltons comprised about 75.5% of the total virion protein and is believed to be the capsid protein. The other two protein components are likely to be internal proteins. DNA polymerase found associated with purified KRV prefers native, double stranded DNA as a template. Maximal activity occurs at pH 9.0 and 0.003 M $MgCl_2$. The enzyme is inhibited by EDTA, KCl and p-chloromercuribenzoate. All four deoxynucleotide triphosphates are required for maximal activity. The requirements for a native DNA template and inhibition by p-chloromercuribenzoate are properties that distinguish KRV-DNA polymerase from the DNA polymerase isolated and purified by Kornberg.

KRV-DNA extracted by both phenol and 2% SDS is a linear single-stranded molecule. The linear configuration of the DNA is also supported by the fact that it is hydrolysed by exonuclease I. As measured in the electron microscope, the DNA is 1.5 ± 0.206 μ in length. This is the first documented report of a linear single-stranded DNA molecule.

Significance to Bio-medical Research and the Program of the Institute:

Kilham rat virus (KRV) is a parvovirus, a group of small (15 to 30 nm) DNA containing animal viruses. KRV was first isolated from a rat sarcoma. It can produce an acute fatal illness, mental retardation and specific deformaties of growth and development when injected into newborn hamsters. KRV is related antigenically to another single stranded DNA containing parvovirus, the H-1 virus. H-1 virus was isolated from a human transplantable tumor. These small DNA containing viruses are interesting since they contain only enough DNA to code for about 3 proteins. With this small amount of information, KRV is able to replicate in an infected cell and kill the cell. Because of its small size, KRV could serve as an important model in studying the virus-cell interaction.

Proposed Course of the Project:

1. To investigate further the DNA polymerase found associated with KRV. Attempts will be made to solublize the enzyme separating it from other proteins and nucleic acid. The enzyme will be characterized, its requirements for maximal activity determined and the enzyme products studied. The role of the enzyme in virus replication will be investigated.

2. To study the replication of KRV-DNA in the infected cell. The viral single-stranded linear DNA may have double stranded and/or a circular configuration during replication. These possibilities will be investigated. If a double stranded KRV-DNA intermediate is involved in replication it should be possible to determine if the viral specific RNA is synthesized from the input viral DNA strand or the replicate strand.

Honors and Awards:

Invited lecture: Department of Microbiology, Johns Hopkins University, April, 1970.
Putnam Memorial Hospital Institute for Medical Research. Bennington, Vt., May, 1971.

Publications:

Salzman, L. A. and White, W. L.: Structural proteins of Kilham rat virus. Biochem. Biophys. Res. Commun. 41: 1551-1556, 1970.

Salzman, L. A. DNA polymerase activity associated with purified Kilham rat virus. Nature, in press.

Salzman, L. A., White, W. L., Kakefuda, T.: Linear, single-stranded DNA isolated from Kilham rat virus. J. Virol., in press.

Serial No. NIAID-84B
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies with Isolated Mammalian Metaphase Chromosomes

Previous Serial Number: Same

Principal Investigators: Dr. Robert S. Lake
Dr. Norman P. Salzman

Other Investigator: Mrs. Jo Goidl

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	20/12		12/12	32/12
Professional:	14/12		12/12	26/12
Other:	6/12			6/12

Project Description:

Objectives:

To obtain metaphase chromosomes free of cellular components.

To characterize the proteins associated with different chromosomes using immunologic and chemical procedures. To see whether there are different proteins associated with different chromosomes, and to establish the immunological interrelationship between proteins of human and other animal species.

To produce antiserum specific for one or a fraction of total human chromosome complement, and to use this reagent to screen for chromosomal translocations.

Methods Employed:

Metaphase chromosomes are isolated from cultured mammalian cells using a variety of methods at neutral or acid pH. Chromosomes are purified by sedimentation through high density sucrose and colorimetric tests used to determine macromolecular content (DNA, RNA and protein). Acid-soluble proteins are extracted with 0.25 N HCl and analyzed by electrophoresis on polyacrylamide containing 6.25 M urea at pH 2.7 with acetic acid. Acid-insoluble proteins are extracted with 2% SDS, 1 M urea and the SDS-proteins separated from DNA by warm phenol extraction. Resulting proteins are separated on discontinuous Tris-glycine-SDS polyacrylamide gels.

Major Findings:

Three major groups of chromosomal proteins have been selectively extracted from Chinese hamster metaphase chromosomes. These are the non-histone acid soluble proteins (ca. 20 proteins), the histones (5 major proteins) and the acid-insoluble proteins (ca. 45 proteins). Acid insoluble proteins range in molecular weight from 15,000 to 180,000 daltons. The non-histone proteins have been found to be glycoproteins as determined by their incorporation of ^{3}H-glucosamine. By comparing the number and types of proteins in chromosomes with those in other nucleoproteins, metaphase chromosomes are found to be enriched in various non-histone acid soluble species and acid insoluble species, but to contain similar histone complements when compared with purified chromatin.

Significance to Bio-medical Research and the Program of the Institute:

A thorough cataloging of nuclear proteins unique to metaphase chromosomes will facilitate an understanding of their structural and/or functional roles in the control of genetic expression. With knowledge of the normal composition of chromosomes, both the genesis and maintenance of genetic abnormalities can be meaningfully analyzed. Such understanding can be extended to virus-host cell interactions where major changes are manifested in the cell nucleus leading to chromosomal aberrations or oncogenic cell transformation.

Proposed Course of the Project:

Examination of the physical and chemical properties of chromosomal constituents will continue. Now that all Chinese hamster chromosomal proteins have been catalogued and partially characterized it should be possible to preparatively separate individual proteins of interest for immunological analysis. Specific antisera will be used to determine the unique location or distribution of the various proteins in chromosome size groups.

Honors and Awards: None

Publications: None

Serial No. NIAID-84C
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanisms of DNA Replication and Integration

Previous Serial No.:

Principal Investigators: Dr. N. P. Salzman
Dr. E. D. Sebring
Dr. T. Steimatsky
Mrs. M. Thoren

Other Investigators: None

Cooperating Units: Dr. T. Kelly, LVD, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	42/12		12/12	54/12
Professional:	24/12		12/12	36/12
Other:	18/12			18/12

Project Description:

Objectives:

To define in molecular terms the mechanism of SV-40 DNA replication and the process by which SV-40 DNA becomes integrated into the cell genome and to understand mechanisms which control expression of the integrated viral genome.

Methods Employed:

SV-40 infected cells have been fractionated to effect a separation of cellular and viral DNA. Radioactive precursors have been incorporated into replicative intermediates (RI's) of SV-40. RI has been examined by electron microscopy, isopycnic centrifugation, and velocity rate sedimentation in neutral and alkaline conditions. Immunofluorescent tests have been used to detect the T and V antigens of SV-40.

Major Findings:

1. The Structure of the SV-40 DNA Replicative Intermediate has been defined.

By electron microscopy and biochemical analysis the structure of replicating SV-40 DNA has been established. Newly replicated DNA is not covalently linked to the parental SV-40 DNA template. This finding excludes one current model proposed for replication of covalently closed circular DNA, the rolling circle model. In addition, there is no covalent link between the two newly replicated DNA strands. Eighty to ninety percent of the isolated replicating molecules contain superhelical regions which indicates that a swivle is absent in the replicating molecule. Replicating molecules with these unique features have not been described previously. It has been possible to effect a fractionation of these molecules based on the extent to which they have replicated. The newly replicated DNA can then be isolated from the replicating molecules by alkaline sedimentation. By this procedure, SV-40 sequences which correspond to limited regions of the SV-40 genome can be obtained. These fragments are of great value in locating various gene functions within the viral genome. (Sebring, Kelly, Thoren and Salzman).

2. Factors that Control SV-40 DNA Expression have been examined.

A restriction enzyme isolated from E. coli B will cleave SV-40 DNA comp. I at a single site. The product of this cleavage is a linear 14S SV-40 DNA molecule (SV-40, Comp. III). The infectivity of this linear DNA has been examined, as has its ability to induce T-antigen synthesis and the synthesis of viral coat proteins (V antigen). These properties have been compared with SV-40 Comp. I and Comp. II. Comp. III has only 1/100 the infectivity of Comp. I and II. However, it is as effective as Comp. I and II in inducing the synthesis of T and V antigens. The finding of reduced infectivity combined with the ability to effectively stimulate V antigen synthesis poses some interesting new questions. It is possible that transcriptional and translational controls which normally couple the synthesis of V antigen to viral DNA synthesis are abolished when a linear rather than a circular DNA molecule is used to initiate an infectious cycle. (Steimatsky, Sebring and Salzman).

Significance to Bio-medical Research and the Program of the Institute:

Integration of viral DNA into the cell genome is likely to occur with a number of viruses and is probably important in latent viral infections. These studies hope to explain viral latency and also to ellucidate critical steps in viral transformation by oncogenic viruses.

Proposed Course of the Project:

The site of initiation of SV-40 will be determined. The role of viral and cellular enzymes in SV-40 DNA replication will be defined.

Honors and Awards:

Reappointed Editor of Journal of Virology, 1971-1975.
Appointed member of the Virology Study Section, 1971-1975.

Invited Lectures at:

1. Univ. of Virginia School of Medicine, Charlottesville, Va.
2. Second Lepetit Colloquium, Paris, France
3. Florida State Univ., Tallahassee, Fla.
4. Univ. of Pittsburgh, Pittsburgh, Pa.
5. Cold Spring Harbor Lab., Cold Spring Harbor, N. Y.

Publications:

Salzman, Norman P.: Biochemical aspects of viral transformation. In Vitro, Journal of the Tissue Culture Association, Vol. 6, 349-354, 1971.

Serial No. NIAID-85A
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure of SV-40 DNA

Previous Serial Number: Same

Principal Investigator: Dr. Frank DeFilippes

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	24/12			24/12
Professional:	12/12			12/12
Other:	12/12			12/12

Project Description:

Objectives:

Production of defined nucleotide sequences of SV-40 DNA and elucidation of their function.

Methods Employed:

Definite double stranded sequences of SV-40 DNA are produced by the action of restriction enzymes (deoxyribonucleases) obtained from E. coli and H. Influenzae. Further size reduction is achieved by use of exonuclease III of E. coli which digests the single strands of the duplex DNA from opposite ends of the molecule. The DNA is then denatured to produce single strands which are characterized by sedimentation and gel electrophoresis.

Major Findings:

SV-40 DNA circles are cleaved across both strands in the same local region by the restriction enzymes from either E. coli B or E. coli K lysogenized by P1 phage. Neither enzyme produces a significant amount of acid soluble DNA products after prolonged incubation. The B enzyme needs S-adenosylmethionine (SAM) and ATP as cofactors and the P1 enzyme is enhanced by SAM and ATP as originally reported for the enzymatic digestion of lambda phage DNA.

When the linear DNA produced by the coli restriction enzyme is treated with exonuclease III and the single strands of the DNA are tested, the limit product has about one half the original length. Thus no large duplex piece of SV-40 DNA remains resistant to attack by exonuclease III after the initial cleavage by the coli restriction enzymes.

The restriction enzyme from H. Influenzae produces smaller pieces of SV-40 DNA than the coli enzymes. In addition, this enzyme cleaves all SV-40 DNA I circles while digesting with the B and P1 enzymes always show a small surviving fraction of resistant circles. Sucrose gradient sedimentation indicates three size classes of single stranded DNA are produced by the Hemophilus enzyme and as with the B and P1 enzymes no extremely small pieces are produced.

SV-40 DNA circles which are isolated at pH 12.0 and neutralized are proper substrates for all classes of restriction enzyme which indicates that at least the sites of enzymatic attack remain as ordered structures after exposure to high pH.

Significance to Bio-medical Research and the Program of the Institute:

Production of defined pieces of SV-40 DNA may lead to the structure of the genetic element causing malignant transformation of cells.

Proposed Course of the Project:

The loss of biological activity of enzymatically degraded SV-40 DNA can be followed by infectivity tests as well as antigenic tests for T and V antigens. A correlation between the size of the DNA after enzymatic treatment and the surviving biological activity will resolve the SV-40 genome.

Honors and Awards: None

Publications: None

Serial No. NIAID-87A
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Properties of the Nucleic Acid and Protein Components of Adenovirus-Associated Viruses

Previous Serial Number: Same

Principal Investigator: Dr. James A. Rose

Other Investigators: Dr. Barrie J. Carter
Mr. Frank Koczot

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	20/12		12/12	32/12
Professional:	4/12		12/12	16/12
Other:	16/12			16/12

Project Description:

Adenovirus-associated viruses (AAV) are a recently recognized group of defective animal viruses. Productive AAV infection occurs only when cells are also infected with an adenovirus. Four AAV serotypes are now known. We have shown previously that AAV DNA is segregated in a way not as yet observed with any other virus. Single, complementary DNA strands are separately encapsidated in different virions. On extraction of viral DNA, the complementary strands anneal to form duplex molecules. There are two species of complementary DNA strands which can be separated in CsCl density gradients. Studies are directed toward the biological and biochemical characterization of AAV infection.

Objectives:

1. To determine the base composition of each complementary strand species of AAV-DNA.

2. To determine the base composition of *in vivo* synthesized AAV RNA.

3. To determine whether one or both complementary strand species serves as a transcriptional template *in vivo*.

Methods Employed:

The AAV serotypes are produced in human kidney cells (monolayer) and KB cells (spinner culture). Viruses are purified in CsCl density gradients, and DNA is extracted from purified virus by treatment with proteolytic enzymes, sodium dodecyl sulfate and phenol. The complementary strand species are separated by centrifuging denatured, bromodeoxyuridine-substituted DNA to equilibrium in neutral CsCl density gradients. For base composition determinations, ^{32}P-labelled DNA and RNA are hydrolyzed, and the mole fraction of each base calculated from high voltage electophorograms. DNA/RNA complementarity is determined by nucleic acid hybridization and hybrids are detected on nitrocellulose filters or in CsCl density gradients.

Major Findings:

1. The AAV DNA strands which are more heavily substituted with bromodeoxyuridine band at a greater density in CsCl and would thus be expected to contain more thymidine. This was confirmed by direct measurement of the base composition of each strand species. The "heavy" strands contained 20% more thymidine than the "light" strands. Complementarity of the strand species was reflected by their A:T equivalence.

2. *In vivo* synthesized AAV-specific RNA (RNA which hybridizes with AAV DNA) has a base composition like that of the "light" DNA strands. This suggests that the "heavy" strands are the transcriptional template *in vivo*.

3. *In vivo* synthesized AAV-specific RNA hybridizes only with "heavy" strands which confirms the template function of this strand species. Tentatively, these strands can be designated "minus" strands, while the non-transcribed, replicated "light" strands are designated "plus" strands.

Significance to Bio-medical Research and the Program of the Institute:

Human infection with AAV has been shown to be frequent, although a virus-specific clinical disease has not been defined. The significance of human AAV infection is not known. Characterization of biochemical and biological properties of these viruses may be eventually important with respect to human disease. The mechanism(s) by which adenoviruses assist AAV replication are relevant to understanding natural modes of viral infection and may be important with respect to the development of biochemical controls of virus replication.

Proposed Course of the Project:

Continue to study AAV replication and defectiveness.

Honors and Awards: None

Publications: None

Serial No. NIAID-87B
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Properties of Adenovirus DNA

Previous Serial No.: Same

Principal Investigators: Dr. James A. Rose
Dr. Thomas J. Kelly, Jr.

Other Investigators: Dr. Suzanne Polmar

Cooperating Units: Dr. Marc S. Lewis, NINDB

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	16/12			16/12
Professional:	10/12			10/12
Other:	6/12			6/12

Project Description: Physical and biochemical cahracterization of the adenovirus genome.

Objectives:

1. Application of physical and biochemical techniques for sizing and mapping the adenovirus genome.

2. To investigate adenovirus DNA transcription in vivo.

Methods Employed:

Viruses are produced in KB cells and purified in CsCl equilibrium density gradients. Viral DNA is extracted from purified virus by treatment with proteolytic enzymes, sodium dodecylsulfate, and phenol. DNA molecular weights are determined by the high speed equilibrium technique of Yphantis, sucrose sedimentation, and by molecule length as measured from electron-micrographs. Electron-microscopic mapping techniques are used to obtain a physical map of selected adenovirus genomes.

Major Findings:

1. Intergeneric hybrids between adenovirus and SV40 genomes may be useful models for studying the mechanism of DNA integration as well as the genetic determinants of viral oncogenesis. We have found that electron-microscopic mapping techniques can be used to analyze the detailed structure of hybrid DNA from these animal viruses. Based on electron-microscopy, the structure of an Ad.7-SV40 hybrid genome ($E46^+$ PL1) can be reconstructed as follows: (1) the hybrid genome contains 16% less Ad.7 DNA than the non-hybrid Ad.7 genome. (2) this deletion consists of the segment of DNA mapping between 0.05 and 0.21 molecular lengths in the non-hybrid Ad.7 DNA molecule. (3) the deleted DNA has been partially replaced by an amount of heterologous DNA equivalent to 75% of the complete SV40 genome.

2. The representation of SV40 DNA sequences in an Ad.7-SV40 hybrid genome ($E46^+$ PL1) was estimated by competitive DNA/DNA hybridization. Based on this technique, it was found that the hybrid genome contained 45-50% of sequences present in the complete SV40 DNA molecule. Coupled with mapping data (above) this finding suggests the possibility that a repetitive segment of SV40 DNA sequences may be included in the SV40 DNA which is integrated into the Ad.7 genome.

Significance to Bio-medical Research and the Program of the Institute:

Further definition of structural and functional characteristics of adenovirus DNA may lead to some understanding of the molecular basis of viral carcinogenesis and add to knowledge of the interactions between virus and cell during viral infection.

Proposed Course of the Project:

1. Continue to map segments of viral DNA by electron-microscopic techniques.

2. Investigate the genetic functions of physically defined segments of viral DNA.

3. Continue to investigate the *in vivo* transcription of adenovirus DNA to gain understanding of control mechanisms for virus synthesis.

Honors and Awards: None

Publications:

Kelly, T. J., Jr. and Rose, J. A.: SV40 integration site in an adenovirus 7-SV40 hybrid DNA molecule. *Proc. Natl. Acad. Sci.* 68: 1971, in press.

Serial No. NIAID-87C
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Properties of Herpesvirus DNA

Previous Serial No.: Same

Principal Investigator: Dr. James A. Rose

Other Investigators: Dr. Alan S. Rabson, Lab. of Pathology and Biology, NCI

Cooperating Units: Dr. Robert W. Chambers, Dep't. of Pathology, Georgetown University Medical School

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	8/12			8/12
Professional:	4/12			4/12
Other:	4/12			4/12

Project Description: Physical and biochemical characterization of herpesvirus genomes.

Objectives:

1. Measurement of genetic relatedness among human herpesviruses.

2. Test human cells for the presence of herpesvirus genes.

Methods Employed:

Viruses are produced in HEP2 and WI-38 cells and concentrated by differential or continuous flow centrifugation. DNA is recovered from virus preparations by treatment with proteolytic enzymes, sodium dodecylsulfate (SDS) and phenol. Further purification of viral DNA is achieved in CsCl density gradients. Cell DNA is extracted by methods similar to those used for recovery of viral DNA. Cell RNA is purified by phenol extraction, alcohol precipitation, and treatment with deoxyribonuclease. Assessments of genetic relatedness and detection of virus-specific nucleic acid are based on DNA/RNA and DNA/DNA hybridization reactions utilizing nitrocellulose filters.

Major Findings:

Techniques for purification of herpesviruses have been improved. Initial virus precipitation with PEG 6000 and a combination of rate and density sedimentation steps yields highly purified virions.

Significance to Bio-medical Research and the Program of the Institute:

Certain herpesviruses are known to be important human pathogens. In addition, there is evidence that the herpes-like particle (EB Virus) which has been associated with human lymphoma cells is related to the infectious mononucleosis agent. Whether or not EB virus is an etiologic agent for human lymphomas is not known. The detection of herpesvirus genes in certain human cells might indicate their possible role in human oncogenesis or perhaps lead to some understanding of the mechanism of chronic viral infection.

Proposed Course of the Project:

1. Continue genetic relatedness studies among members of the herpesvirus group.

2. Test human cells for the presence of herpesvirus DNA and RNA.

Honors and Awards: None

Publications: None

Serial No. NIAID-88
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Synthesis of Proteins in SV-40 Infected Cells

Previous Serial number: Same

Principal Investigator: Dr. Stanley Barban

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	36/12			36/12
Professional:	12/12			12/12
Other:	24/12			24/12

Project Description:

Objectives:

1. To further characterize the structural proteins of purified SV-40 virions.

2. To compare the protein composition of SV-40 mutant strains which differ in plaque-type and other biological properties and relate these properties to the physical and chemical constituents of the virions.

3. To determine the role of the viral genome in directing the synthesis of both viral and non-structural viral proteins in infected cells.

Methods Employed:

SV-40 virus was grown in primary and continuous African green monkey kidney cell cultures and purified by a sequence of CsCl density gradient and equilibrium centrifugations. Radioactively labeled virus was prepared by propagating the virus in cell cultures containing either ^{3}H or ^{14}C mixtures of amino acids. The protein structure of the virus was studied by dissociating isotopically labeled and unlabeled purified virus preparations with either sodium dodecyl sulfate, alkaline buffers, or 8M urea, and fractionating the sub-units by zonal centrifugation, polyacrylamide gel electrophoresis and isoelectric focusing in gels, sucrose density and urea gradients. Specific viral proteins were further identified by immunological methods.

Major Findings:

We have previously shown that SV-40 virus particles contained a minimum of four polypeptide components. Molecular weights of these viral polypeptides were estimated to range from 45,000 to 16,000 daltons. The major structural component was the capsid protein consisting of two subunits. Comparison of a large-plaque morphology SV-40 mutant with a small plaque virus strain by double-isotope labeling revealed a generally similar polypeptide pattern by polyacrylamide gel-electrophoresis.

Employing the technique of electrofocusing, which involves separation of proteins of different isoelectric points by electrophoresis in a pH gradient, a distinct difference between the capsid proteins of the SV-40 mutants could be demonstrated. By this technique, the other protein components of these mutants were found to be very similar. Isoelectric focusing of a mixture of capsid proteins from both large and small-plaque SV-40 mutants labeled with different isotopes revealed a charge difference of less than 0.10 pH unit. This result suggests a difference in either amino acid composition or protein conformation of the capsid proteins of these plaque morphology mutants.

Significance to Biomedical Research and the Program of the Institute:

Detailed knowledge of the role of the SV-40 tumor virus in directing its own biosynthesis and to interaction with the metabolism of its host cell is of obvious basic and practical significance to the problems of oncogenesis and control mechanisms of virus-cell metabolism and viral chemotherapy.

The finding of a difference in the capsid proteins of SV-40 mutant strains possessing different morphological and biological properties is of fundamental significance in future genetic and immunological studies of mutant virus strains.

Proposed Course of the Project:

Efforts will be made to isolate and further characterize the coat proteins of the SV-40 mutants by peptide mapping, amino acid composition, and immunological analyses. The synthesis of SV-40 structural proteins and other macromolecules at different times during virus replication will be studied and comparisons made using the SV-40 virus mutants.

Honors and Awards: None

Publications:

Barban, S. and Goor, R. S.: Structural proteins of Simian Virus 40. J. Virol. 7: 198-203, 1971.

Serial No. NIAID-88A
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: DNA-RNA Hybridization between SV-40 Specific RNA and Chromosomal DNA from SV-40 Transformed Chinese Hamster Cells

Previous Serial Number: Same

Principal Investigator: Dr. Cephas T. Patch

Other Investigators: Dr. Norman P. Salzman
Mr. Michael Radonovich

Cooperating Units: Dr. Arthur S. Levine, Medicine Branch, NCI

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	32/12			32/12
Professional:	14/12			14/12
Other:	18/12			18/12

Project Description:

Objectives:

The objective of this work is to prepare DNA from fractionated chromosomes of SV-40 transformed Chinese hamster cells and to test this DNA for SV-40 homology by RNA-DNA hybridization.

Methods Employed:

Cell Cultures. Explants from various tissues of Chinese hamsters were cultured. A fibroblast from heart tissue proved to be an excellent cell line. Ten cultures of this cell line were independently exposed to high multiplicities (about 100 PFU/cell) of SV-40 (SV 222, SP 3V3). The virus exposed cells were cultured for several weeks and cloned. Eleven clones were isolated from five of the mass transformed cultures.

Chromosome Fractionation. Cells are cultured to 80-90% confluence and vinblastin sulfate added to a concentration of 0.2 µg/ml. The cells are incubated for 10 hours, harvested with trypsin, and suspended in hypotonic citrate to a concentration of 3×10^6 cells/ml. After 20 minutes the cells are broken by shaking vigorously with acetate buffer, pH 3. The chromosome suspension is fractionated by layering onto a 10-40% sucrose gradient in pH 3 buffer and centrifugation for 20 minutes at 1200 RPM in an A-XII zonal rotor.

After centrifugation about 35 fractions are collected. Samples of each are stained and examined microscopically for chromosome size distribution. Based on the microscopic examination, the chromosome fractions are pooled, pelleted and resuspended in tris-eota, pH 8.2 to protect the DNA from nucleus action. The pooled chromosomes are assayed for DNA by the Bunton modification of the diphenylamine test.

DNA-RNA Hybridization. The purification of chromosomal DNA and the hybridization experiments are carried out in the laboratory of Dr. A. Levine. In general, however, DNA is prepared by the method of Marmur. SV-40 specific radioactive RNA is made *in vitro* using RNA polymerase from *E. coli*. Hybrids are formed by incubation in 2 x SSC at 65°C for 20 hours. Hybrids are collected on nitrocellulose filters, washed, and counted.

Major Findings:

Two technical problems, fragmentation of DNA, which may occur as a result of breaking metaphase cells at low pH and contamination of chromosomes with non-chromosomal DNA of interphase nuclei, have been studied.

During the chromosome isolation procedure employed in this work the molecular weight of purified chromosomal DNA is reduced to about 10^6 as measured by neutral and alkaline sucrose gradients. The mechanism of DNA breakdown at low pH is not clear but may be due to acid depurinization with subsequent breakdown at those sites. Attempts to demonstrate depurinization by labeling with (H^3) adenine and (C^{14}) thymidine and measuring the H^3/CM ratio have been unsuccessful. However, using sucrose gradients as a criterion it can be shown that DNA fragmentation is minimized by working at low temperature and keeping the time of exposure to pH 3 as short as possible. By using this modification in the procedure, DNA was purified from fractionated chromosomes of one line of transformed cells. In this experiment the viral homology appeared to fractionate with intermediate sized chromosomes. This cell line produced low levels of infectious virus. Since free viral particles may lead to a false interpretation subclones were rendered free of infectious virus by culturing in SV-40 antisera and studies with DNA prepared from fractionated chromosomes of virus-free cells are being carried out. In addition, transformed lines are being subcloned in order to obtain a clone that will yield chromosomes free of any interphase nuclear DNA.

Significance to Bio-medical Research and the Program of the Institute:

In order to understand more completely the molecular biology of viral transformation, it is desirable to know whether the viral homology associated with DNA from transformed cells is physically associated with host chromosomes, and, if so, whether it is localized in a single sedimentation group of chromosomes.

Proposed Course of the Project:

If SV-40 homology can be demonstrated to be localized in a specific sedimentation group of metaphase chromosomes similar experiments will be performed using several independently transformed cell lines to determine whether the same integration site is always involved.

If the experiments described above are successful in demonstrating a localized site of integration of the SV-40 genome in transformed Chinese hamster cells, the same technique can be applied to determining the site of integration of polyoma DNA in doubly transformed cell lines. Similarly, other virus-host systems can be studied.

Honors and Awards: None

Publications: None

Middle America Research Unit

Balboa Heights, Canal Zone

Summary 1

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PHS-NIH
Summary Statement
Middle America Research Unit
Balboa Heights, Canal Zone
July 1, 1970 through June 30, 1971

Karl M. Johnson, M.D., Director

Dr. Ihor Zachary completed a two-year assignment as Staff Associate and Drs. William C. Reeves, Jr., and David H. Martin entered on similar status during the year. Mr. Charles Twigg returned to Bethesda after four years during which he established a sound data processing system that serves all of our research and administrative operations. With the arrival of Mr. Ross Buckwalter, biostatistian, the capability of the laboratory to plan studies and analyze results was greatly increased. Elsewhere, however, we were unable to add any more scientists, a situation that represents a significant relative decline in overall effectiveness because we were unable to exploit several exciting lines of investigation that have been developed over the past half-decade. Dr. Peter Franck, in charge of work on Venezuelan encephalitis, accepted a position in our Institute in Bethesda. By chance alone, we were able to fill this gap with the temporary assignment of Lt. Col. Gerald Eddy, D.V.M., Ph.D., of the U.S. Army, thus preserving our competence in this area at a time when the disease is epidemic in a new region of the hemisphere and threatens to enter the continental United States.

Our program of informal training in virology and cell culture technics was continued. Five scientists and technicians from Latin American laboratories spent a total of 14 man-months during the year. Active collaborative research on genetic aspects of animal and human infection was carried out in our laboratories by Dr. Uzi Ritte, Smithsonian Institution post-doctoral fellow, and by Dr. Carmelo Ciniglio, Department of Biology, University of Panamá.

Laboratory research programs were divided into five principal areas, with continued emphasis on elucidation of the ecology of infection by appropriate field and experimental studies. Drs. Webb and Johnson studied the biology of Machupo virus infection in its natural rodent host, Calomys callosus. Dr. Peters investigated clinical hepatitis and Australia antigenemia in several tropical communities, and Drs. Tesh and Chaniotis expanded their work on virus infections transmitted by phlebotomine sandflies, particularly vesicular stomatitis. Work on Venezuelan encephalitis was vigorously pursued by Drs. Eddy, Martin, Peralta, and Walton. Leishmaniasis was the principal subject of work by the parasitology group led by Drs. Burke and Collins.

Research Highlights

Tacaribe Viruses. Long-term evaluation of our rodent control program in Magdalena, Bolivia, disclosed that despite continued differential trapping pressure exerted upon the Machupo virus reservoir Calomys callosus, this species appears to be spreading in the town and now outnumbers the common house mouse Mus musculus. This surprising development has forced re-evaluation of our concepts concerning interaction of the two species. We now must alter the program to attempt general rodent control—which will be more costly and carries the risk of an "ecological vacuum" effect.

A peculiar, limited but virulent, outbreak of hemorrhagic fever was investigated in Bolivia. Five of six patients died, transmission was definitely by direct contact and occurred geographically and ecologically outside the known endemic area, and at least three patients had gross hepatitis. Convalescent sera from the single survivor had group-reactive complement-fixing antibodies, but no definite type-specific Machupo virus neutralizing activity. We suspect that a new serotype may have produced this outbreak and will attempt to characterize the agent if a safe laboratory is made available.

Early results of a long-term study of host response to Machupo virus infection suggest that ability of adult Calomys rodents to develop persistent tolerance without detectable circulating antibodies is determined genetically as an autosomal recessive trait. We also found complete correlation between tolerant infection and development of a severe microcytic, hypochromic anemia with reticulocytosis and splenomegaly. As far as we know, no virus has ever been discovered to have both of these potentials. This model system therefore appears to have very important bio-medical potential.

Venezuelan Encephalitis. Despite a thorough, effective vaccination campaign in equines that halted overt disease in southern Nicaragua at the end of 1969, an epizootic occurred during the summer of 1970 in adjacent Costa Rica. We had just completed a baseline survey of humans, equines, and mosquitoes in the exact area when the first cases were reported. Thus, for the first time, we were able to show that (1) equine mortality from the disease was independent of sex or age, (2) human infection ceased shortly after equine infection—which in this case was abruptly halted by vaccination, and (3) that the crabhole mosquito Deinocerites pseudis was probably involved in transmission as evidenced by 12 virus isolations from this species.

Three new laboratory markers were devised to differentiate the epizootic virus from a strain widely enzootic in Central America. The distinct nature of the equine and human virulent agent was repeatedly confirmed. Experimental confirmation of differential virulence of these viruses for equines was obtained. The epizootic virus produced much more viremia and frequent encephalitis. The appearance of central nervous system disease was always correlated with detectable neutralizing antibodies, suggesting that encephalitis may be an immunopathologic rather than a direct viral syndrome.

Bovines were shown incapable of sustaining viremias sufficient to infect mosquitoes. The attenuated vaccine (strain TC-83) was found to produce minimal viremia in horses, was highly immunologic in several well-designed field trials, and failed to induce abortions in pregnant mares.

Taken together, these data provide a strong basis for veterinary and public health action this year in the southwestern United States. The virulent virus is currently active in Mexico less than 250 miles from Texas. It will cross the border this summer. Our data, supported by that of other research groups, indicates that equines should be vaccinated widely in the path of the epizootic. Failure to do so will almost surely result in many hundreds of horse deaths—and the occurrence of encephalitis in humans. The practical rewards of our work on this problem during the past decade are ready to be harvested.

Hepatitis. Three further examples of possible venereal transmission of Au-antigen associated hepatitis were documented. A correlative study of Au-antigen and IgM immunoglobulin response in clinical hepatitis was completed. The inverse relationship previously noted in small groups of children inoculated with Au-positive and Au-negative "hepatitis" blood was confirmed in the general population. Age and sex were not important variables. Thus IgM levels in conjunction with Au-antigen assays offer a better means of epidemiologic and clinical separation of long and short incubation hepatitis.

Persistent Au-antigenemia was documented among Guaymi Indians of western Panamá. Antigenemia appears to be "familial" but not clearly compatible with an inherited autosomal recessive gene. A variety of genetic markers are being measured in an effort to confirm family histories and to find a correlate of the chronic antigenemic state.

Vesicular Stomatitis Virus (VSV). Several more isolations of the virus were made from wild sandflies last year, principally from *L. trapidoi*. Another species *L. ylephiletrix* was shown capable of experimental transmission of the virus. No new clues were uncovered as to the vertebrate reservoirs of the virus in nature. Major progress was made, however, in rearing sandflies in the laboratory, and it was found that larvae actually eat the leaves of certain plants found in the forest. An exciting development was the demonstration of transovarial transmission of VSV by *L. trapidoi*. The virus was detected in 15-20% of individuals in each stage of development after female flies were fed on viremic infant hamsters. Since the life cycle required about 8 weeks, and since there was a definite quantitative decrease in virus concentration in the pupal stage followed by an increase in emergent adults, replication rather than mere survival of the virus seems assured. Detailed quantitative experiments to elucidate the biological parameters of this phenomenon are in progress.

Parasitic Diseases. Cutaneous *Leishmaniasis* continued to be seen in the Canal Zone last year. Of 28 confirmed cases, 26 were seen in military personnel. In previous years most cases were associated with training exercises on the Atlantic side of the Zone and at the Rio Hato base in interior Panamá. With the closing of Rio Hato and shift in training locale to the Empire Range area on the west bank of the Panama Canal, Pacific side, a completely different pattern emerged in that 18 of the 28 cases were referable to this area. Most of these cases were treated with a pentavalent antimonial drug and are being followed culturally and serologically.

The cotton rat *Sigmodon hispidus* is under study as a laboratory host for *Leishmania* infection. Most fresh isolates from humans induced definite ulcerative dermal lesions in this animal with an average incubation interval of only 25 days. Infected animals developed circulating antibodies as measured by indirect fluorescence. Studies on the role of lymphoid cells, antilymphocyte serum and antibodies on the course of infection are in progress. Despite the lack of dermal sensitivity to crude *Leishmania* antigen, it was found that animals simultaneously inoculated with organisms and lymphoid cells from previously infected rodents developed ulcerative lesions much more rapidly than controls. Sigmodon thus appear to offer an important host system for experimental and perhaps epidemiological (possible sentinel animal) study of this disease.

Work on Toxoplasmosis, particularly the biology of oocysts was continued. Oocyst formation was documented in the native feral cat Felis jaguarundi, the first species other than the house cat to exhibit this pattern. Repeated attempts to demonstrate oocyst formation in house cats on second oral infection were unsuccessful—and the challenge interval was taken up to six months. That this immunity was "intestinal" was shown by an experiment in which cats were infected subcutaneously with Toxoplasma trophozoites. Two months later, when all animals had fluorescent antibody titers of 1:32 or greater, they were challenged orally and all responded with oocyst formation that was quantitatively similar to that noted in control animals. That oocysts may represent the most infectious form of the parasite for man was supported by the occurrence of clinical disease in a laboratory technician who had worked more than five years with trophozoites without becoming infected.

Serial No. NIAID-100-A
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Surveillance of Infectious Disease Situations to Determine Investigative Potential for MARU
Previous Serial Number:	Same
Principal Investigators:	Dr. K. M. Johnson and Lt. Col. J. C. Burke
Other Investigators:	Dr. P. H. Peralta, Dr. P. H. Webb, Dr. R. B. Tesh, Dr. T. E. Walton, Lt. Col. G. A. Eddy, Dr. D. H. Martin, and Dr. W. C. Reeves
Cooperating Units:	Ministries of Health in Panamá, Guatemala, Bolivia, Ecuador, Honduras, Nicaragua and Costa Rica PAHO/PASB/WHO, Zone III Office, Guatemala Health Director, Canal Zone Office of the Chief Surgeon, USARSO, Canal Zone Gorgas Memorial Laboratory, Republic of Panamá Canal Zone Military Dispensaries (U.S. Army, Navy, and Air Force) Hospital del Niño, Republic of Panamá Hospital Division, Chiriqui Land Company, Panamá Hospital Roosevelt, Guatemala Laboratorio de Salud Publica, Nicaragua Hospital Infantil Maria Teresa, Guatemala Laboratorio Veterinario, Ministry of Agriculture, Republic of Panamá

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	1 6/12			1 6/12
Professional:	8/12			8/12
Other:	10/12			10/12

Project Description:

Objectives:

1. To provide for prompt recognition of changes in human or animal morbidity and mortality which might lead to further investigations of research interest:

a. By prospective surveillance programs.
b. By investigation of individual cases or epidemics in progress in the field.
C. By retrospective serum surveys of human and animal populations.

2. To maintain contacts with various health agencies in the Canal Zone, Republic of Panamá, and throughout Central America in order that promising situations may be learned of and investigated in the shortest possible time.

3. To gather sufficient information concerning an epidemiologic situation to:

a. Pursue studies in depth as a separate project.
b. Refer to some other agency.
c. Drop from consideration.

Methods Employed:

Prospective studies include clinical surveillance for cases of acute neutropenia at Gorgas Hospital, and the collection and testing of cord blood samples obtained from several local hospitals for immunoglobulin levels.

When leads of importance are obtained in the course of testing clinical case specimens or through information supplied by cooperating agencies, arrangements for field activities are made. In the Canal Zone, these are made through the Canal Zone Health Bureau and Gorgas Hospital; contacts are also maintained with the Office of the Chief Surgeon, U.S. Army Southern Command, and clinical facilities of individual military units. Throughout Central America and Panamá, contacts with individual Health Ministries are initiated and maintained with the cooperation of the Pan American Health Organization Zone III Ofice in Guatemala.

Laboratory procedures include attempted isolation of the agents involved by inoculation of material into appropriate cell cultures and laboratory animals. Standard serologic procedures including in some cases fluorescent antibody techniques are used to test for specific serum antibodies.

Field procedures include specialized epidemiologic, clinical, entomologic, and biologic techniques.

Major Findings:

An unusual epidemic of hemorrhagic fever in Bolivia was reported to us by Dr. R. H. LeBow, a USPHS Peace Corps physician. Detailed investigation of this outbreak described in Project No. NIAID-100-B of this report revealed the possibility of a new Tacaribe complex serotype as cause of the cases. If confirmed, we will expand ecological work on the problem in Bolivia.

Umbilical cord blood IgM levels reflect in utero exposure to infectious agents. In the United States there appears to be an inverse correlation between such immunoglobulin levels and socioeconomic status. Panama City, which has hospitals serving relatively well-defined socioeconomic segments of society, provided the opportunity to examine this relationship in a tropical environment. Forty samples were obtained from babies born at each of two hospitals in the city. Higher IgM levels were found at the charity hospital than at the insurance-operated social security institution.

Late in 1970, when Gorgas Hospital acquired a Coulter Model S counter for routine hematology testing, a new pattern of study in outpatient clinics was established. Nearly every patient with any conceivably infectious illness now has a complete blood count performed. Relatively large numbers of instances of neutropenia were found among children. A cooperative surveillance program has been established with the departments of Pediatrics and Pathology in order to follow the incidence of acute neutropenia over a full year, to carefully follow up all patients and to attempt etiologic diagnosis of these cases.

Significance to Bio-Medical Research and the Program of the Institute:

This program continued to provide medical research intelligence indispensable to the long-range functioning of this unit, while offering pertinent services to the various countries within our geographic locale.

Proposed Course of the Project:

The hemorrhagic fever materials will be processed in a special laboratory in the United States where protection of personnel against the hazard of infectious aerosols is assured. The cord blood study will be expanded into a laboratory project in order to learn more about the potential organisms responsible for the elevated IgM levels and to observe developmental parameters of the children. The neutropenia study will be evaluated for potential inclusion in our program at the end of the 12-month surveillance interval.

Serial No. NIAID-100-B
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Tacaribe Group of Viruses

Previous Serial Number: Same

Principal Investigators: Dr. P. A. Webb and Dr. K. M. Johnson

Other Investigators: Dr. N. H. Wiebenga, Dr. C. J. Peters, Mr. J. Vogel

Cooperating Units:
Viropathology Unit, Center for Disease Control, Atlanta, Georgia; Dr. Frederick A. Murphy
Arbovirus Infections Unit, Center for Disease Control, Atlanta, Georgia; Dr. C. Calisher
USAMRU Panama; Lt. Col. S. C. Benbrook
Instituto Nacional de Laboratorios de Salud, La Paz, Bolivia; Dr. L. Valverde Chinel

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	6 4/12			6 4/12
Professional:	2 4/12			2 4/12
Other:	4			4

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PH43-67-59	K.M.Johnson	Smithsonian	C.O. Handley,Jr.	$6,720

Project Description:

Objectives:

1. To characterize and determine the natural relationship of viruses of the Tacaribe group by study of their immunological, morphological, and biophysical properties, the bionomics and pathogenesis in animals, particularly in the natural rodent reservoir of Machupo virus, *Calomys callosus*.

2. To develop a safe, effective vaccine for Bolivian hemorrhagic fever.

3. To continue limited routine epidemiological surveillance in certain areas of the Department of Beni, Bolivia, and to study more intensively areas where evidence of Machupo virus activity is high.

4. To make available reagents and provide facilities for diagnosis of viruses in this group to other North and South American investigators and to assist them in active control measures to contain new outbreaks of human disease.

Methods Employed:

Standard virus isolation and serologic techniques were employed, principally the use of cell cultures, fluorescent antibody determinations, immuno-electrophoresis, animal inoculation, and standard pathologic sectioning and staining. Virus-infected organs were sectioned and fixed at MARU and submitted to CDC for electron microscopic study. Field collections were made in Cochabamba, Bolivia, where recent outbreaks of human disease had occurred. Mammal skins and skulls were submitted to the U.S. National Museum, Smithsonian Institution, for identification.

Major Findings:

Epidemiology of BHF: In late January 1971 a student nurse in a hospital at Cochabamba, Bolivia (elevation 9,000 feet) died after a 10-day febrile illness diagnosed as "paratyphoid fever." When four secondary cases occurred among her contacts, we were asked to investigate. Before we had finished, a tertiary case occurred in the pathologist who performed an autopsy on one of the secondary cases, the city of Cochabamba and the entire country of Bolivia was gripped by panic, and the following picture emerged: (1) The student nurse spent Christmas holidays at her home—a ranch in Beni Province about 50 miles outside the known BHF endemic zone—and sickened immediately after her arrival back at the hospital. (2) Five of the six patients died. (3) At least three patients had objective clinical evidence of acute hepatitis, never before seen with BHF. (4) Isolation attempts using well-preserved splenic tissue from one of the cases failed to yield a Tacaribe-complex virus. (5) Rodent trapping at the ranch of the index case revealed no Calomys callosus, the only known reservoir of BHF. (6) Convalescent sera from the only survivor showed high complement-fixing (CF) antibody titers for Machupo and Junin viruses, but neutralization tests showed only equivocal Machupo antibody levels.

We concluded that the possibility of a new Tacaribe serotype cannot be ruled out. In view of the extremely high mortality observed in this outbreak, we strongly feel that isolation and characterization of this agent should be done only in a laboratory offering "class 3" protection for personnel—regardless of history of past Machupo virus infection. A collaborative study is planned with USAMRIID, U.S. Army Medical Research and Development Command, Fort Detrick, Maryland.

Follow-up serologic investigation of all known patient contacts in Cochabamba and the Beni is in progress—but probably will prove to be negative.

Biology of Tacaribe Viruses: A large scale attempt to genetically select subcolonies of Calomys callosus respectively immuno-competent and immuno-tolerant to Machupo virus infection is in progress. Preliminary results suggest that tolerant-tolerant matings produce almost 100% tolerant offspring, whereas competent-competent pairings result in a significant fraction of tolerant offspring. It is too soon, however, to conclude that Machupo tolerance is a genetically controlled, single locus, autosomal recessive trait.

Work on this problem may be expedited by the recent findings that immunotolerance is accompanied by a significant anemia. All newborn Calomys infected with Machupo virus not only are immuno-tolerant, but also develop major splenomegaly and an anemia marked by hypochromia, reduced mean corpuscular volume, and persistent reticulocytosis. The process may be hemolytic. Animals inoculated as adults and divided into competent and tolerant by virologic means were found to faithfully show the same correlation in hematologic tests—tolerants having anemia and large spleens, competents having neither. Anemia can be reliably measured four weeks after virus infection and may serve as a cheaper, more rapid marker for tolerance than the viral tests that require 3-4 months for definitive classification of a grown animal.

The discovery of this major virus-induced anemia prompted investigation of some other parameters as possible pre-infection markers of the tolerant versus competent response. To date these include hemaglobin and red cell esterase electrophoretic studies. Calomys callosus fetal hemaglobin was detectable for only 2 days after birth, and preliminary work suggested no other differences in either normal animals or virus-infected ones that fit the observed requirements. A modification in technique, however, has permitted the separation of one band into two components which require re-examination.

Esterases appear to be more promising. There are major changes in normal animals beginning the second week of life and several distinct "normal" patterns. Furthermore, there are serial changes in esterases in rodents infected by Machupo virus, with a suggestion of some correlation with the type of infection induced.

A small colony of Calomys callosus from the Santa Cruz region of Bolivia has been established. Rodents from this region previously yielded Latino virus, a Tacaribe-complex agent nonpathogenic for man. We plan to study the biology of Latino and Machupo viruses in these animals to compare responses with those observed in San Joaquin rodents of the same species.

Miscellaneous Findings: Data obtained from Calomys callosus rodents captured in Beni, Bolivia, in 1969 and 1970 were analyzed and two interesting facts emerged: (1) There was a high correlation between spleen weight and isolation of Machupo virus. Over-all frequency was about 20%, but spleens of 0.2 g or greater were 75% positive; those between 0.15 and 0.2 g, 30% positive; and those less than 0.15 g, only about 1% positive. Thus the experimentally observed biology is confirmed in nature and spleen size does serve as a convenient simple marker of virus presence in this species. (2) When virus prevalence in Calomys rodents was correlated with history of human hemorrhagic fever, it was found that places where disease had occurred within one year of rodent capture 35% of animals were positive. In contrast, places free of human disease 3 years or longer had only 5% positive rodents. This is our first evidence in support of a hypothesis that Machupo virus prevalence bears some kind of direct relationship to rodent population density and that the genetic interaction between rodent and virus will prove to be important not only in determining when and where human disease occurs, but also plays a role in population regulation of the rodent.

Significance to Bio-Medical Research and the Program of the Institute:

The ability to recognize and characterize arenaviruses was again demonstrably important as shown by the 1971 hemorrhagic fever in Bolivia. The peculiar features of this outbreak suggest that a new pathogenic virus may be responsible— lending further urgency to the complex problem of definitive vaccine development.

The mission of the Institute is research on infectious diseases of public health importance. Biological studies of the arenaviruses have already and will continue to shed light on some fundamental problems in human medicine. What are the factors responsible for differential human virulence of these viruses, what are the mechanisms behind specific immunological tolerance, how do these viruses induce anemia? Are there human counterparts for the recognized rodent-associated arenaviruses? This project has now presented the Institute with an outstanding opportunity for a major, productive, goal-oriented research effort. The question is whether the advantage will be seized!

Proposed Course of the Project:

A major medical problem experienced by one of the senior investigators currently threatens the entire project at MARU. For the next few months, at least, work will be limited to continued efforts to attenuate Machupo virus for the marmoset and to keep the Calomys virus-genetics study functioning. Hemorrhagic fever materials from Bolivia, 1971, will be studied at Frederick, Maryland, if suitable arrangements can be made. What really is needed is a laboratory and a scientific staff capable of sustained work on the arenaviruses under maximum biohazard security. Potentially available facilities of this type exist only in Frederick, Maryland.

Publications:

Justines G., and Kuns, M. L.: Matucare virus, a new agent recovered from *Ornithodoros* (*Alectrobius*) *boliviensis*. *Am. J. Trop. Med. & Hyg.*, *19*: 697-702, 1970.

Johnson, K. M.: Fiebres hemorrágicas de Sur America. *Medicina* (Buenos Aires), *30* (Suppl. #1): 99-110, 1970.

Webb, P. A., Johnson, K. M., Hibbs, J. B., and Kuns, M. L.: Parana, a new Tacaribe-complex virus from Paraguay. *Arch. fur die Gesamte Virusforschung*, *32*: 379-388, 1970.

Murphy, F. A., Webb, P. A., Johnson, K. M., Whitfield, and Chappell, W. A.: Arenoviruses in Vero cells: Ultrastructural studies. *J. Virol.*, *6*: 507-518, 1970.

Johnson, K. M.: Hemorrhagic Fever, Transmission Unknown: Hemorrhagic fever in South America: Argentine and Bolivian hemorrhagic fever. In Beeson, P. B. and McDermott, W. (Eds.): *Cecil-Loeb Textbook of Medicine*. New York, W. B. Saunders, 1971.

Johnson, K. M.: Hemorrhagic Fever. Transmission Unknown: Epidemic hemorrhagic fever: Hemorrhagic nephroso-nephritis. In Beeson, P. B. and McDermott, W. (Eds.): *Cecil-Loeb Textbcok of Medicine*. New York, W. B. Saunders, 1971.

Casals, J., Henderson, B. E., Hoogstraal, H., Johnson, K. M., and Shelokov, A.: A review of Soviet viral hemorrhagic fevers. *J. Infect. Dis.*, *122*: 437-453, 1970.

Serial No. NIAID-100-C
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Studies on Arboviruses
Previous Serial Number:	Same
Principal Investigators:	Dr. P. H. Peralta and Dr. T. E. Walton
Other Investigators:	Dr. K. M. Johnson, Dr. R. B. Tesh, Lt. Col. G.A.Eddy, Dr. D. H. Martin, and Miss G. Oro
Cooperating Units:	Ministries of Health and Agriculture in Costa Rica, Nicaragua, Honduras, El Salvador, Guatemala and Panamá University Autónoma de Honduras; Dr. Manuel Figueroa, Head, Department of Microbiology Instituto Nacional de Salud, Colombia; Dr. H. Groot Virology Unit, Fort Detrick, Maryland; Drs. McKinney, Spertzel, and Kahn Arbovirus Unit, CDC, Atlanta Georgia; Dr. B.Henderson and Dr. W. D. Sudia Gorgas Memorial Laboratory; Dr. P. Galindo and Dr. A. Adames Yale Arbovirus Research Unit; Dr. R. E. Shope Cornell University Medical College, Department of Microbiology; Dr. W. Scherer and Dr. R. Dickerman MAG-FAO; Dr. R. Horrell, Ing. O. Miranda OIRSA, Costa Rica; Dr. W. Eskew Veterinary Laboratory, Ministry of Agriculture,Panamá Dr. O. Alvarez Universidad del Valle, Colombia; Dr. C. Sanmartin

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	8			8
Professional:	4			4
Other:	4			4

Project Description:

Objectives:

To develop methods for measuring arboviruses and their antibodies; to study in detail the pathogenesis, epidemiology, and ecology of various arthropod-borne virus infections in man and animals; and to define the antigenic relationship of members of selected serologic groups or complexes within the arbovirus class.

Methods Employed:

Standard field and laboratory procedures are used for isolation of viruses and detection of antibodies in man and a wide variety of animals. A longitudinal study approach is applied in certain instances to the study of various natural populations.

Major Findings:

Venezuelan Equine Encephalitis (VEE). In last year's annual report, the use of the Fort Detrick attenuated live vaccine (TC-83) in Nicaragua during the 1969-1970 epizootic was mentioned. Although 93% of 89 horses (Nicaraguan horses) developed neutralizing antibodies to the vaccine strain of VEE virus, only 65% of these animals produced neutralizing antibody to the strain isolated during the 1969-70 Central American epizootic. The significance of these and similar findings with other groups of horse and human sera are now under study as part of the programs to be discussed later.

In spite of the thorough vaccination campaign in Nicaragua, an outbreak of VEE occurred in Costa Rica in August 1970. Serologic studies in both man and horses were initiated prior to and during the epizootic. Included was another field evaluation of the TC-83 VEE vaccine. All of 55 vaccinated horses developed neutralizing antibody to the vaccine virus. In addition, horse deaths on this ranch stopped abruptly at 8 days after vaccination.

Further studies have been conducted with the vaccine virus. Eight horses experimentally vaccinated never had viremias greater than $10^{2.5}$ $TCID_{50}$/0.1 ml and all became seropositive. Four horses were clinically ill with signs of anorexia and listlessness; seven developed fevers and all exhibited leukopenia. Another group of 20 horses were vaccinated in the Canal Zone and none became clinically ill or viremic; 70% developed neutralizing antibody to the homologous group.

One group of 8 horses and two groups of 4 horses have been experimentally infected with the antigenic subtype I-E (Mena II) and two I-D subtypes (3880 and Magengue). None of these animals were clinically ill, viremias were low or did not occur and all animals produced neutralizing antibodies to the homologous virus. It is our opinion from these data that the enzootic strains are relatively apathogenic for Equidae and less pathogenic than the vaccine strain which was derived from a virulent isolate (Trinidad donkey #1). This explains the earlier queries regarding lack of equine disease in spite of the known endemnicity of VEE in many areas of Central America. For these enzootic agents, the horse apparently serves neither as an amplifer of the virus nor a sentinel for their presence.

In contrast, three groups of 4 horses have been experimentally infected with epizootic VEE strains, MF-8 (Honduran isolate of the I-B subtype), V-676 (Venezuelan I-C) and San Pelayo (Colombian untyped). In all cases, horses were viremic, clinically ill with generally severe disease or death, and produced neutralizing antibodies. Viremias have been shown to range above $10^{5.5}$ SMIC LD_{50}/0.1 ml in nearly every case during the period of detectable viremia. The histopathological changes of the central nervous system and various organs have been studied for all animals.

Epidemiologic studies carried out during the Costa Rican outbreak revealed the following: Thirteen cases of VEE were identified among a human population of 109 by serologic conversions for an attack rate of 12%. When horse deaths ceased, presumably secondary to vaccination, human VEE infection was halted. A breakdown of the population by age and sex revealed that the attack rate was significantly lower among children under 8 years of age. No other differences were noted. Of the 13 cases, six had a severe flu-like syndrome with neurological symptoms and VEE virus was isolated from the blood of four of these. Three gave histories of mild nonspecific flu-like illness and four gave no history of illness whatsoever. Four months after cessation of equine deaths in the region, we returned and rebled 78 individuals who had been previously serologically negative. Only one was found to have converted. This constitutes positive epidemiological evidence for what had been previously suspected: Prevention of the disease among equines serves to protect the human population.

Among a population of about 120 horses surveyed in this area, mortality rates of 38% were found. The rate would undoubtedly have been higher had not all equines in the area been immunized with the Fort Detrick attenuated vaccine. No significant differences could be found in horse mortality rates between sexes and between various age groups, although there was a suggestion that perhaps other animals are more severely infected. Serological studies of these specimens revealed that a significant percentage were susceptible to infection at this point and the total cessation of equine deaths within 10 days of vaccination constitutes good evidence for its effectiveness.

Eighteen virus isolations were made from equines in the course of these studies. All but one were taken from animals without obvious signs of disease. Seven sick animals with encephalitic symptoms did not have virus in their blood, but were positive for virus-neutralizing antibody. This observation raises the question of the possible role of immunological mechanisms in the pathogenesis of this disease in equines.

With regard to the question of other possible hosts in maintaining and spreading epizootics, two groups of four cows were infected with San Pelayo and MF-8 isolates. Although all animals exhibited seroconversion to the VEE strains, none were detectably viremic (using our technics) and none were clinically ill. It would appear, then, that the bovine plays an insignificant role in the epizootiology of VEE. The isolation of VEE virus from one calf in Guatemala during the I-B epidemic (Sudia et al.) in the face of this study suggests that viremia occasionally occurs in the cow. Although this information is not immediately available, the viremia was probably below the suggested mosquito-infection threshold.

In an attempt to elucidate the method of transmission of the VEE strain involved in the current VEE epizootic, we conducted a serologic study of dogs along the southern Nicaraguan Pacific coast. Based upon earlier studies after the epizootic's southernmost extention to the Costa Rican border, we believed that these species were good sentinels for the presence of VEE. All equines in this area had been vaccinated and no horse deaths had been previously reported in this area. A previous serologic survey among equines in this area showed that VEE was not endemic there. We found a significant number of antibody-positive animals near the coast and also discovered several cases of equine deaths in the area since January 1, 1971 (the epizootic in this area of Nicaragua was halted in 1969 by a massive vaccination campaign). In one horse which was recently ill, but recovered, high titers of neutralizing antibody were found in a pattern suggesting infection with the epizootic strain. In addition, CF antibody which is known to persist after VEE infection for relatively short periods of time, was demonstrable. We feel that the evidence is good that the virulent epizootic strain of VEE was or is active in this area. This indicates that it may be transmitted in certain locations without susceptible equines to act as an amplifying host. Such modes of transmission are well known for the relatively avirulent endemic strains, but this would be the first time evidence has been presented in favor of similar mechanisms among the virulent strains.

Sandfly-associated Viruses. As an outgrowth of the project on ecology of vesicular stomatitis virus (VSV) (Project NIAID-104-B), an urgent need for preparing reagents and tooling up to identify sandfly isolates became apparent as more and more non-VSV strains were recovered. Therefore, virus pools and serologic reagents were made this year for over a dozen prototype sandfly-associated viruses—members of the Sandfly Fever group, members of the Changuinola group, and ungrouped Pacui virus. Included were Chagres and Changuinola prototypes, first isolated at MARU, for which new reagents were made, and most of the known members of the Sandfly Fever group of which the majority were new to our experience.

The need for capability in identifying sandfly isolates has led us into an investigation of the serologic relationship among members of the Sandfly Fever group, as demonstrated by plaque neutralization (N) using constant virus and varying serum dilution. Knowledge of the N relationships within the group heretofore has been based on log neutralization index determinations in suckling mice (with undiluted antibody reagents). For this group, complement fixation (CF) is specific only and the more group-reactive HI test suffers from great sensitivity to nonspecific inhibition.

Study of the Changuinola group has been resumed. Reagents have been or are being made to all 1959-1961 Changuinola isolates from Panamá and Irituia virus. It is hoped that using Vero cell and hamster materials and plaque neutralization, it may be possible to delineate the individual serotypes within the group (for which the CF test is only a group-specific test) and thus arrive at a basis for identifying isolated members of this group.

Studies on Dengue Viruses. Progress with the several dengue virus serotypes was quite limited. So far, seven dengue strains and three viremic sera (representing types 2, 3, 4, and 6) provided by WRAIR, as well as two prototype dengue viruses (types 1 and 5) already in our hands were inoculated into suckling mice intracerebrally and into Vero cell cultures in tubes. Most caused illness and death in mice in the first trial, and many caused clear-cut CPE in first passage in Vero. As time permits, passages will be continued, reagents for the sufficiently pathogenic strains prepared, and plaque neutralization test relationships within the dengue subgroup of arbovirus group B studied.

Significance to Bio-Medical Research and the Program of the Institute:

Studies of the recent VEE epidemic in Central America are of importance in indicating areas requiring future investigation, and also demonstrate exciting new and previously unknown possibilities in the epidemiology of this economically significant disease. We believe that most epidemics have their origin in overflow from contiguous sylvan endemic foci, but our finding of introduction of virus from one geographic region to a susceptible population 2,000 miles distant raises a host of new questions of great practical importance. A search for endemic areas and susceptible equine populations near and within the continental United States is now of greater immediate significance. Increasing populations of man and domestic animals, greater transportation and progressive man-made changes in the ecology of Central and South America may make future epidemics more frequent and more extensive.

The epidemiology of VEE may soon become the immediate concern of public health officials in the United States if the current epizootic continues its northward movement through Mexico. Methods of control and protection of the human populations apparently resides in the ability to immunize susceptible equines. Much more work is urgently needed to determine whether equine immunization will solve the public health problem; if so, whether presently available vaccines are safe and effective; and if not, whether vaccines acceptable for direct human use in selected high-risk populations can be developed. Studies of the ecology of the virulent viruses will prove important in attempting any form of eradication once they have entered a susceptible area.

Each of the studies included in the project contributes to our continuing broad efforts to understand the mechanisms of pathogenesis, the properties and the natural behavior of arboviruses pathogenic for man in the Americas, and thus a foundation for making future public health decisions.

Proposed Course of Project:

VEE Viruses. More detailed studies of the pathogenesis of encephalitis in equines are planned. These will include assessment of cross-protection conferred by infection with several of the presently recognized sero-subtypes. Work will be initiated to determine whether the TC-83 vaccine virus is capable of reversion to virulence after serial equine passage. We also plan to investigate the potential of several natural and laboratory derived virus strains as candidate live vaccines for equines. An attempt will be made to determine whether the virulent virus subtype I-B is now endemic in Central America.

Other Arboviruses. We plan to pursue the epidemiology of sandfly viruses, using the many isolates so far obtained and the stored serums and data on wild and domestic animals and man obtained in the course of our work on vesicular stomatitis viruses.

Dengue virus studies will be pursued with the ultimate aim of testing selected sera from the INCAP-ICNND collection (Project NIAID-102) against a number of serotypes, to determine whether any evidence can be found for the presence in Central America or Panamá, in the past, of any dengue serotype other than type 2, and to see if any dengue infection has occurred in the past 15 years.

Publications:

Franck, P. T. and Johnson, K. M.: An outbreak of Venezuelan encephalitis in man in the Panama Canal Zone. Am. J. Trop. Med. & Hyg., 19: 860-865, 1970

Honors and Awards:

Dr. T. E. Walton:

Invited to present a paper on "Encefalomielitis Equina Venezolana en América Central. Cuadro Epidemiológico actual y possibilidades para el control." III Congreso de Medicina Veterinaria de Centroamerica y Panamá; San Salvador, El Salvador, April 1971.

Became a Diplomate of the American College of Veterinary Microbiologists, November 1970.

Discussant of a paper on "Epizootiology of the Feline Respiratory Viruses" at a Symposium for Selected Feline Diseases, sponsored by Am. Vet. Med. Assoc.; Ithaca, New York, September 1970.

LTC Gerald A. Eddy

Invited to present a paper on "Overall View of Venezuelan Equine Encephalitis in the Americas" at an International Roundtable on VEE sponsored by Pan American Health Organization; Mexico City, D.F., May 1971.

Dr. K. M. Johnson

Invited speaker at annual meeting of American Public Health Association, Houston, Texas, October 1970. Subject presented, "Another Mouse That Roared."

Invited participant in the PAHO/WHO International Conference on the Application of Vaccines Against Viral, Rickettsial, and Bacterial Diseases of Man. Held in Washington, D.C., December 1970.

Consultant to the Instituto de Investigacion Clinica, Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela. To help establish a longitudinal study of Venezuelan encephalitis activity in the Province of Zulia, Venezuela. March 1971.

Invited participant on the Panal on Venezuelan Equine Encephalitis at the IV Inter-American Meeting on Foot-and-Mouth Diseases and Zoonoses, and sponsored by PAHO, in Lima, Peru, April 1971.

Invited to accept a two-year term on the Editorial Board of the American Journal of Tropical Medicine and Hygiene, beginning January 1971.

Serial No. NIAID-100-D
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Korean Hemorrhagic Fever

Previous Serial Number: Same

Principal Investigator: Dr. N. H. Wiebenga

Other Investigator: Dr. K. M. Johnson

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	9/12			9/12
Professional:	6/12			6/12
Other:	3/12			3/12

Project Description:

Objectives:

1. To demonstrate and characterize an infectious agent as the etiology of epidemic hemorrhagic fever that occurs in the Republic of Korea.

2. To define the immunoglobulin response in patients admitted to a U.S. Army hospital with the diagnosis of Korean hemorrhagic fever (KHF).

Methods Employed:

Since the etiology (presumably a virus) of KHF has defied detection by many and various efforts, our general approach is limited to new techniques or laboratory hosts that were not used in previous isolation studies. Most important, we believe, is the inoculation of cell cultures with acute-phase clinical specimens obtained in an Army hospital in Korea from 1967 through 1968. The inoculated cultures will be tested by the indirect fluorescent antibody test (IFAT) using convalescent sera obtained from the same patients. This effort will primarily rely on Vero and human embryo kidney cell cultures, but other cell types may be employed. As available, small numbers of unusual laboratory animals will be tested. Immunoglobulins in convalescent sera will be assayed in commercially supplied radial diffusion plates.

Major Findings:

Isolation Studies. As a result of a recent equipment failure, all of the material collected in Korea and all of the material prepared and under study at MARU were lost. The extent of loss has been verified by failure to recover infectivity from known stock of Tacaribe group viruses stored within the equipment that failed. The results of our isolation study are now of no importance.

Serum IgM levels. Earlier studies had indicated some reaction with Latino virus in the indirect fluorescent antibody test and reactive sera were all obtained during the period of time associated with increased levels of IgM. We extended the IgM studies done in Japan by selecting those patient collections that had serial serum specimens, at weekly intervals for four or more weeks. As before, many sera taken during the first week of illness showed their maximum titers and then demonstrated a rising titer during the second week of illness and these are in reserve for further testing by the IFAT. The work was delayed by technical difficulties in producing good quality coverslips infected with Latino virus and by the losses during mechanical failure of the Revco. However, these losses can and will be replaced.

Significance to Bio-Medical Research and the Program of the Institute:

Recent discussion with Professor Lee from Korea indicate that there was increased incidence of the disease during last winter, although total incidence is not known because KHF is not a reportable disease. It is of interest that the distribution of cases was more widespread than usual and larger numbers of civilian cases were observed. Professor Lee Ho has plans now to intensify their studies with emphasis on isolation of the etiologic agent. The nature of the IgM response and its possible relationship to Latino virus remains of importance.

Proposed Course of the Project:

Evaluation of the acute-phase clinical specimens indicates that they are no longer suitable for isolation studies. Fortunately, the serial serum samples were stored elsewhere and they are in good condition. These will be used for further study of the IFAT reaction with Latino virus, especially hoping to demonstrate whether or not the reaction is due to IgM antibody.

Serial No. NIAID-101
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hepatitis and Australia Antigen in Panamá

Previous Serial Number: Same

Principal Investigators: Dr. C. J. Peters and Dr. W. C. Reeves

Other Investigators: Dr. K. M. Johnson, LTC S. C. Benbrook, Mr. J. Vogel, and Mr. Ross Buckwalter

Cooperating Units:
Ministry of Health, Republic of Panamá
Hospital del Seguro Social, Republic of Panamá
Hospital del Niño, Republic of Panamá
Hospital Santo Tomás, Republic of Panamá
Gorgas Hospital, Canal Zone
Office of the Chief Surgeon, United States Army, Southern Command
Dr. Carmelo Ciniglio; University of Panamá
Laboratory of Viral Diseases, NIAID, NIH; Dr. Robert H. Purcell and Dr. Jerrold J. Lander; Project Serial No. NIAID-70-D

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	4 2/12			4 2/12
Professional:	2 8/12			2 8/12
Other:	1 6/12			1 6/12

Project Description:

Objectives:

1. To investigate the epidemiology of Au antigen and acute hepatitis in Panamá.

2. To study the genetics and epidemiology of the carrier state for Au antigen in a detailed longitudinal study among the indigenous peoples of Panamá.

3. To inoculate certain Panamanian primates with Au antigen, based on their availability and relevance for the epidemiology of Au antigen in Panama.

4. To evaluate the relevance of serum immunoglobulin levels to the study of hepatitis.

5. To evaluate the relation of certain chronic infections to infections by cytomegalovirus (CMV), Epstein-Barr (EB) viruses, and the presumed agent of one type of hepatitis as measured by Au-antigen and -antibody techniques.

Methods Employed:

Au antigen was determined by gel diffusion (GD), complement fixation (CF), and immunoelectrophoresis (IEOP). Immunoglobulin concentrations were determined by radial immunodiffusion using commercial antibody-in-agar plates. CMV and EB antibodies were determined by the CF and indirect fluorescent antibody tests (IFAT) respectively.

Major Findings:

1. Au-negative hepatitis was shown to be associated with a marked increase of serum immunoglobulin (IgM) concentration. No evidence for the presence of monoclonal or low-molecular weight IgM could be obtained. Au-positive hepatitis patients did not have significant elevation of their IgM levels and showed a decrease in serum IgG concentration during the acute phase of their disease. Rising IgM levels were seen in a subject with acute Au-positive hepatitis progressing to chronic hepatitis. No increase in IgM was seen in one Au-positive subject with a relapse nor in four Au-negative subjects with relapses. Normal IgM levels were found in subjects with drug-induced hepatic necrosis.

There were no differences in IgM, IgG, IgA, or IgD immunoglobulin levels in Guaymi Indian carriers of Au antigen and matched controls.

2. The NIAID reference antiserum was tested against a variety of local Au-antigen-containing sera and shown to be a useful reagent for CF testing. About 2,000 sera were selected from the total of 5,000 sera previously tested by gel diffusion and screened by CF. The increased sensitivity of the CF test was confirmed, but general patterns of occurrence of Au antigen were the same. The IEOP test has been instituted, using a locally prepared guinea pig antiserum and has provided a rapid, sensitive test for screening which is not affected by anticomplementary activity.

A guinea pig antiserum suitable for complement-fixation and fluorescent antibody work has been prepared and tested.

3. The epidemiological data gathered from 69 consecutive Au-positive hepatitis patients was analyzed. Au-positive hepatitis was not recognized among children. About one-third of the cases were associated with transfusion, narcotics use, or occupational exposure to human blood. Nine of the cases were presumably due to person-to-person contact. Presumably the contact must be intimate, since six of the nine were sexual partners, and no evidence for other infections was found among the remaining family contacts.

4. Frozen glycerolated red cells, red cell hemolysates and further serum samples have been obtained from Guaymi Indian families. These have confirmed the 4% prevalence of Au antigenemia in healthy Indians. Repeat bleedings have shown that antigenemia persists for at least a year. Au antigenemia has a familial pattern, but does not fit any simple Mendelian model of inheritance. In conjunction with Dr. Carmelo Ciniglio, preliminary testing of phosphoglucomutase types has been carried out.

5. All primate sera from the MARU serum bank (500 sera) were tested for Au by gel diffusion and found to be negative. Marmosets, night monkeys and spider monkeys were inoculated with Au-antigen-positive human sera and serial bleeding tested by gel diffusion without detecting Au antigen or Au antibody. Histological examination of liver tissue was negative.

6. A cluster of 8 cases of hepatitis in an apartment building in Panamá was investigated. The cases were all in children, all Au positive, and thought to be acquired by environmental fecal contamination.

7. In conjunction with Drs. Jerrold Lander and Robert Purcell of NIAID, 900 sera from hepatitis patients, contacts, and Panamanian Indians have been tested for Au antibody by radioimmunoassay. A selection were also tested for Au antigen.

Significance to Bio-Medical Research and the Program of the Institute:

The practical importance of the elucidation of the mode of transmission of Au-positive hepatitis is enormous. It is likely that increasing medical administration of blood products and illicit parenteral narcotics use will place all aspects of hepatitis research into a position of priority. The significance of Au antigenemia in population groups, such as the Guaymi Indians, is totally unexplored even though they stand on the threshold of assimilation into general society. The theoretical importance of a disease with features such as protacted incubation period, conversion to chronic degenerative state, existence of a chronic, asymptomatic carrier state, and lack of antibody response detectable by usual tests is great. The analogy to the Machupo-*Calomys* genetic studies and the importance to understanding the mechanism of chronic viral diseases is obvious.

Proposed Course of the Project:

Clinical and epidemiological data from the previous year are being analyzed and testing of specimens for Au antigen completed. The study of hospitalized patients and their contacts will be pursued only if novel situations present themselves or if specific directions are suggested from analysis of current data and radioimmunoassay antibody results.

The guinea pig anti-Au reagent prepared at MARU will be used to prepare a fluorescent antibody reagent to study frozen liver biopsy material and material from experimentally inoculated Panamanian primates.

The study of Au antigenemia among the Guaymi Indians will be continued. Rh antigens, phosphoglucomutase and haptoglobin typing will be used to evaluate paternity in critical families and to assess genetic background of closely related groups with differing frequencies of antigenemia.

Honors and Awards:

Dr. C. J. Peters

Hospital Seguro Social, Panama City, Republic of Panamá: Grand Rounds—presented "Australia Antigen and Hepatitis in Panamá" in Spanish. August 1970.

Hospital Santo Tomas, Panama City, Republic of Panamá: Presented at Grand Rounds "Significance of Australia Antigen for Hepatitis Research and Correlation of Findings in Hospital Santo Tomas"(in Spanish), October 1970.

Gorgas Hospital, Ancon, Canal Zone: At Medical Grand Rounds, presented "Australia Antigen and Hepatitis," September 1970.

Hospital del Niño, Panama City, Republic of Panamá: Presented "Australia Antigen and Hepatitis" at Infectious Disease Conference, February 1971.

Serial No. NIAID-102
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1970

Project Title: Nutritional, Immunological Surveys of Human Populations in Central America and Panamá

Previous Serial Number: Same

Principal Investigator: Dr. K. M. Johnson

Other Investigators:
MARU: Dr. P. H. Peralta and Mr. R. M. Buckwalter.
INCAP: Dr. L. J. Mata

Cooperating Units:
Institucion de Nutricion de Centro America y Panamá, Guatemala
Pan American Health Organization; Pan American Sanitary Bureau; Zone III Office, Guatemala
National Governments of Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, and Panamá

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	3 1/12			3 1/12
Professional:	1 4/12			1 4/12
Other:	1 9/12			1 9/12

Project Description:

Objectives:

To determine prevalence of specific antibodies to a large number of arbo- and other viruses and to certain parasites among families carefully selected on a random basis in each of six countries. These data will then be related to detailed information being collected on nutritional, socioeconomic parameters of these individuals by INCAP and cooperating organizations.

Methods Employed:

Family groups are selected statistically well in advance of actual work. Serum samples are obtained in the towns and villages at the time of large scale clinical and biochemical surveys. Sera are divided with aliquots stored at INCAP and MARU for testing by standard techniques for virus and parasitic antibodies. Complement-fixation testing with exo-antigen from culture forms of Trypanosoma cruzi is used for Chagas' disease testing and the indirect fluorescent antibody test is used for toxoplasmosis. Hemagglutination-inhibition and cell culture plaque neutralization tests are employed for arboviruses. All field data and serum inventories are handled on IBM cards.

Major Findings:

Group B Arbovirus Antibody Survey: Plaque-neutralization (N) tests using microcultures of Vero cells in multi-welled disposable plastic trays were undertaken after it was observed that large numbers of the nutrition survey sera were positive to one or more antigens in hemagglutination-inhibition (HI) tests against 6 different antigens of this serologic group. Last year's preliminary testing indicated that Powassan and Bussuquara viruses could be eliminated from subsequent tests, as viruses that are either absent or of no importance in the areas covered by the serum survey.

This year, the N testing of adult sera was carried out with the four viruses: yellow fever (YF), dengue (represented by strain TH-36), Ilheus, and St. Louis encephalitis (SLE). Since so many of the sera had been found positive in HI tests, representative towns were selected for testing by N method; HI pattern, altitude, and other geographic factors were taken into consideration in making the selection. Adults from 98 of the 159 rural survey communities in the five Central American countries and 22 of the 30 in Panamá have now been tested for N antibodies. At year's end, sera of the children in those communities where adult antibody prevalence is highest are being subjected to N testing, to complete the picture.

Forty-five communities (of which 15 are located in Panamá) showed >50% antibody prevalence against YF virus among adults. About 25 others showed 20-50% prevalence. Vaccination during the last yellow fever epizoodemic in the 1950's is the most likely source of this antibody. Analysis of the data after testing of children's sera has been completed should give an indication of how well humans living in areas where jungle yellow fever might be expected to occur (in any future wave of viral activity) are protected against the virus.

Twenty communities had >30% dengue antibody prevalence among adults, and another five had 20-30%. Thus far, the age of the youngest positive reactor is 18, which indicates that dengue was no longer active after about 1948. Results of testing children's sera should help us to fix the time of last dengue activity with more confidence. Analysis of the completed tests should indicate which areas might be especially vulnerable to invasion by dengue viruses (which have been active in various parts of the Caribbean over the last 10 years).

Twelve towns (four in Panamá) had Ilheus antibody prevalence exceeding 30%, and another six had 20-30%. SLE antibody was found in 20% or more of adults (maximum 40%) in seven towns. Dengue, Ilheus, and SLE viruses have been active on both the Pacific and Atlantic sides of the Continental Divide. Their distribution appears to have been restricted fairly well to certain areas of coastal lowland or to the course of waterways further inland, in focal patterns. Testing of the children should provide an answer to the question of where, if at all, Ilheus and SLE viruses have been recently active in the human population.

HI results correlated fairly well with N data only in Guatemala (mostly negative towns) and Panamá (mostly positive towns), where YF, followed by dengue, HI results were most reliable. In the other countries, the least reliable were the Ilheus HI findings followed by those for SLE virus. Many towns had some Ilheus HI-positive sera, except in Guatemala; these were largely unconfirmable by N test, indicating the widespread occurrence of nonspecific inhibitors that had not been removed by kaolin treatment. Most HI-negative towns probably can be considered N-negative for all practical purposes, since 15 to 45 towns (depending on the virus) with 0 or 1 serum positive by HI were tested by N method and almost invariably found to have 0 or only 1 serum positive by neutralization also.

Significance to Bio-Medical Research and the Program of the Institute:

Ecologic maps of the distribution of human infection with a variety of arboviruses and protozoa having clear-cut present or potential future public health importance in Central America and Panamá are emerging from this project. The survey has provided and is still providing clues as to the factors that govern the transmission of these various infections and as to the sites where we or other research groups may expect to be able to carry out profitable direct investigation of them.

Proposed Course of the Project:

The group B arbovirus neutralization tests are being completed as this year ends. Correlative statistical analyses between antibody pattern and a variety of ecologic parameters are to be concluded for the agents already studied. Tentative conclusions about factors governing transmission of these agents in Central America and Panamá are to be summarized. Manuscripts will be prepared for publication.

Testing of the same serum collection from time to time in connection with other projects (e.g., Studies on Arboviruses, Project No. NIAID-100-C) in regard to virus epidemiology may of course be anticipated, but this project, as such, will probably be terminated this year.

Serial No. NIAID-104-A
1. Middle America Research Unit
2. Parasitology Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Parasitic Infections in Panamá and Central America

Previous Serial Number: Same

Principal Investigator: Lt. Col. J. C. Burke, MSC (Medical R&D Command, U.S. Army)

Other Investigators: Lt. Col. S. C. Benbrook, VC, and Capt. W. T. Collins, MSC

Cooperating Units: Office of Chief Surgeon, USARSO
Military Dispensaries in the Canal Zone (Army, Navy, and Air Force)
Gorgas Hospital, Canal Zone

Man Years:

	USAMRU Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	8 9/12			8 9/12
Professional:	3			3
Other:	5 9/12			5 9/12

Project Description:

Objectives:

1. To clarify routes of transmission, natural reservoir hosts, and pathogenesis of several endemic parasitic organisms.

2. To establish the extent and importance of several of the most significant parasitic infections endemic in this area.

3. To improve methods for diagnosing such infections in the laboratory.

Methods Employed:

The basic techniques are serological testing of human and other animal sera with species-specific antigens in the indirect fluorescent antibody test (IFAT), plus culturing, animal inoculation, and tissue examination to determine infections and host response to organisms causing cutaneous leishmaniasis or toxoplasmosis. Giemsa-stained thick and thin blood smears are examined microscopically for microfilaria, malaria, and trypanosomes.

Major Findings:

Leishmaniasis. Twenty-eight cases of cutaneous leishmaniasis were diagnosed during the past year, 26 of these in military personnel. The majority were recognized between January and March 1971 and all had histories of overnight exposure in tropical forest. Eighteen of these cases were apparently acquired at the Empire Range, a forested training area on the west bank of the Panama Canal near the Pacific terminus. Although this area has been used for many years, recent disease increase is correlated with closure of an army base in interior Panamá and a major increase in extended training exercises at Empire Range. Several other cases were acquired at Fort Sherman, a known endemic area on the Atlantic side of the Canal Zone.

The disease is being treated with a pentavalent antimonial drug. Cultural and serologic studies are being done on all patients to see whether this drug will prove to be better than a previous one employed in more than two dozen cases treated here in the late 1960's.

The cotton rat Sigmodon hispidus is being evaluated as a host for Leishmania infection. Ulcerative dermal lesions grossly similar to those observed in man have been produced with high frequency. Lesions were induced in 28 of 30 animals inoculated with blood agar isolation material taken from 7 patients. Average incubation interval was 25 days. Of five other isolates that had been passaged in blood agar two or more times, only three induced lesions and average incubation was 67 days. Three of nine isolates that had been stored in liquid nitrogen up to one year also induced skin lesions. Preliminary work suggests, also, that pathogenesis of strains is modified by serial passage since the incubation period on the second Sigmodon passage was 122 days.

Several lines of immunological investigation of the disease produced in Sigmodon were pursued. Fluorescent-detectable circulating antibodies appeared in most infected rodents, but required 60-120 days and were generally preceded by the appearance of skin lesions. Peritoneal macrophages from infected and control animals were obtained and inoculated in culture with L. braziliensis leptomonads. Control cells were much more heavily infected than those from "immune" animals. Although leishmaniasis skin tests were uniformly negative in infected Sigmodon, it was found that the inoculation of spleen and thymic cells from infected animals accelerated the dermal response in recipients given organisms with these cells. Two groups of infected animals were given rabbit anit-Sigmodon-lymphocyte serum respectively. Four of 5 of the former and none of the latter group subsequently died. These observations suggest that Sigmodon hispidus may be a powerful tool for the elucidation of host-parasite biology of this important disease.

Toxoplasma gondii. Last year we confirmed the finding of others that orally infected domestic cats produced fecal oocysts which are highly infectious, resistant forms in the life cycle of the parasite and showed that oocysts were readily demonstrable in cats fed freshly isolated Toxoplasma from human cases.

The search for other animals capable of producing oocysts was intensified this year, partly because review of our series of acquired human toxoplasma cases disclosed a very low correlation with presence of even a single cat in the household. Rats were carefully restudied for 28 days following oral infection with negative results. In conjunction with Dr. J. K. Frenkel and Mr. Mark Jewell, University of Kansas, we fed organisms to a variety of wild animals. Kinkajous, olingos, and coatimundis were negative for oocysts, but oocysts were shed for several days by a wild feline, Felis jagaurundi, which subsequently developed circulating antibodies. Oocysts were found also in the feces of an ocelot.

Two further experiments were done with domestic house cats. In the first, several animals that developed oocysts followed by circulating antibodies were refed Toxoplasma 1 to 6 months later to see whether oocysts were again shed in feces. Results were uniformily negative. A second experiment showed conclusively that this immunity was "intestinal" and incidently dashed hopes that cats could readily be vaccinated against becoming oocysts shedders. Three animals were inoculated subcutaneously with trophozooites of the C-37 strain of T. gondii. Two months later, when the animals had fluorescent antibody titers of 1:32 to 1:512, they were challenged by the oral route. All three excreted oocysts in a pattern similar to that of a control animal.

Significance to Bio-Medical Research and the Program of the Institute:

Inability to find non-feline animals capable of excreting Toxoplasma oocysts adds support to the concept that these carnivores are the definitive hosts of this parasite. In temperate areas of the world, this fact, if true, indicates that the focus of future work should be on cat-parasite biology. Initial attempts to "immunize" cats were unsuccessful, but much remains to be done.

The ease of induction of classical skin lesions by L. braziliensis in Sigmodon hispidus provides an important tool which should be exploited in many ways. Both fundamental biology and drug-screening should be advanced rapidly in the next few years.

Proposed Course of the Project:

Toxoplasma immunization of cats will be studied in greater detail. Sigmodon will be used to study differential pathogenecity of a variety of leishmanial parasites—human and nonhuman—and to investigate the immune mechanisms involved in production and resolution of skin lesions caused by L. braziliensis.

Serial No. NIAID-104-B
1. Middle America Research Unit
2. Epidemiology Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ecological Studies of Vesicular Stomatitis Virus

Previous Serial Number: Same

Principal Investigators: Dr. R. B. Tesh and Dr. B. N. Chaniotis

Other Investigators: Dr. K. M. Johnson, Dr. P. H. Peralta, Miss G. Oro, Maj. L. C. Rutledge, and Mr. R. M. Buckwalter

Cooperating Units:
Ministry of Health, Republic of Panamá
Veterinary Section, Ministry of Agriculture, Republic of Panamá
Environmental Health Branch, U.S. Army Southern Command, Canal Zone
University of California, Division of Entomology, Berkeley; Dr. E. S. Sylvester
School of Public Health, University of California, Berkeley; Dr. W. C. Reeves and Dr. C. H. Templis

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	6 2/12			6 2/12
Professional:	2 6/12			2 6/12
Other:	3 8/12			3 8/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PH43-67-59	K.M.Johnson	Smithsonian Institution	C.O. Handley	$6,720
NIH-71-5000C	K.M.Johnson & L.D.Chance	Inter-American Foundation for Tropical Studies, Incorporated	E. L. Tyson	$6,500

Project Description:

Objectives:

1. To study the ecology of vesicular stomatitis virus (VSV) in an endemic area of the disease.

2. To study the biology of phlebotomine sandflies in both field and laboratory settings in order to better understand their role as vectors of VSV and other sandfly-borne agents.

3. To collect and process wild sandflies for the purpose of virus isolation, with subsequent identification and characterization of viruses obtained.

4. To establish laboratory colonies of local man-biting sandfly species in order to investigate their vector potential as well as the mechanism of experimental virus infection and transmission.

Methods Employed:

Standard virus isolation and serologic techniques were employed, using cell culture and animal inoculation. A new microplate precipitin test utilizing specific antisera produced in rabbits and chickens was used to identify blood meals of wild-caught sandflies. Insects used in virus transmission studies were housed in our new insectary. Regular field collections of wild animals and sandflies were carried out at two study sites in the Canal Zone and Panamá under Contract NIH-70-26 with the Inter-American Foundation for Tropical Studies. Results of serologic testing, animal trapping, and daily insect collections were summarized on IBM cards for subsequent tabulation and analysis.

Major Findings:

Viruses Isolated from Sandflies. Regular collections of sandflies for virus isolation and blood meal identification studies were continued at two field stations located near Gamboa, Canal Zone, and El Aguacate, Republic of Panamá. A total of 160,883 wild-caught female sandflies were processed for virus isolation. Nine isolates of VSV-Indiana were obtained; three of the isolations were from pools of *Lutzomyia trapidoi*, a common anthropophilic species in Panamá. In addition to VSV, 237 other confirmed virus isolates were obtained, emphasizing the role of these tiny biting insects in the natural cycles of certain arboviruses. A smaller number of male sandflies were processed and these yielded seven additional non-VSV virus isolates. Preliminary studies were begun to identify and characterize these viruses; most appear to be members of the Phlebotomus Fever and Changuinola arbovirus groups.

Experimental Infection and Transmission Studies. In view of our VSV isolations from naturally infected *L. trapidoi*, attempts were made to establish laboratory colonies of this species, and transmission studies were initiated. Wild-caught *L. trapidoi* were infected with VSV-Indiana by feeding on viremic infant hamsters. It was found that virus titers increased rapidly in infected sandflies and VSV transmission by bite was demonstrated as early as three days after the infective blood meal.

Because of repeated isolations of Phlebotomus-fever group viruses from male sandflies by ourselves and other workers and because of our inability to identify a susceptible vertebrate host which develops a sufficiently high viremia to infect a biting sandfly, the possibility of VSV-Indiana transovarial transmission was investigated. To our surprise and excitement, VSV-Indiana transovarial transmission occurred. In the preliminary studies, it appears that VSV-infected female *L. trapidoi* transmit the virus to about 15 to 20% of their offspring. Virus has been isolated from larvae, pupae, and adults produced by experimentally infected parents. VSV titers in emerging F_1 generation adults are comparable to those found in experimentally and naturally infected female sandflies, suggesting that the F_1 adults could transmit virus by bite to a susceptible host and/or transovarially to their offspring. Studies are now in progress to test these hypotheses and to determine whether transovarial transmission occurs in other sandfly species.

Studies of Sandfly Biology.

1. Natural host preferences of sandflies. Utilizing a newly developed microplate precipitin test, over 3,000 wild-caught, blood-engorged sandflies were tested against class- and order-specific antisera. Most of the sandflies tested fed predominately on mammals, although the frequency of feedings on seven mammalian orders varied with season, species, and locality of the collection. Of 10 sandfly species studied, *L. trapidoi* and *L. ylephiletrix* demonstrated the broadest host range and appear to be of greatest public health importance.

2. A 16-month study of natural population dynamics. This study, which began in 1969, was completed in 1970. Thirty-seven species were identified among 60,455 specimens collected; anthropophilic species comprised 31.2% of the total. The major finding was that sandflies in the tropical forest are perennial with marked seasonal fluctuations; their density changes are related to the amount and distribution of rainfall. Scanty rainfall in the dry season causes a significant decline in the sandfly population. With the onset of rains in May, the population swings upward to peak in late July or early August. Between August and November, the population declines probably as a result of excessive rainfall which waterlogs the ground and kills a large portion of the immature stages. With the cessation of rains, the population attains a secondary peak in late December or early January.

3. Daily and seasonal man-biting activity. This study was also completed in 1970. The daily and seasonal man-biting activity of seven anthropophilic sandfly species were recorded at ground and canopy levels in a tropical

forest over a period of one year. *L. olmeca*, *L. panamensis*, and *L. pessoana* were predominately active at ground level; *L. gomezi*, *L. sanguinaria*, *L. trapidoi*, and *L. ylephiletrix* in the forest canopy. Most sandflies were active primarily between sunset and sunrise. One exception, *L. pessoana*, was found to bite indiscriminately day or night, making it a good potential vector of human disease for persons entering the forest for work or recreation during daylight hours.

4. Daytime resting sites of sandflies. The diurnal resting sites of sandflies in a tropical forest were surveyed. Adult sandflies utilized a wide spectrum of habitats such as tree trunks and buttresses, animal burrows, tree cavities, leaf litter, and green shrubs. Most species showed specific habitat association. Green shrubs and leaf litter on the forest floor were utilized primarily by anthropophilic species.

5. Studies of sandfly flight range and longevity. Utilizing fluorescent powders, a mark-and-release program was recently initiated to determine sandfly flight range and longevity in their natural forest habitat. Preliminary results indicate that these insects are relatively short-lived and rarely travel further than 200 yards from their resting sites. These results are in marked contrast to those obtained in mosquitoes and may yield important new information on the epidemiology of sandfly-transmitted diseases.

6. Colonization and laboratory rearing of local man-biting sandflies. Considerable progress was made in developing techniques for rearing and maintaining sandflies in the laboratory. While we are not yet able to mass produce sufficient numbers of sandflies for laboratory experiments, we can successfully maintain and feed wild-caught insects and rear their offspring with minimal mortality. Work in this field will continue as well as attempts to develop a sandfly tissue-culture cell line.

Cooperative Studies. In trapping and bleeding wild animals to study the ecology of VSV, a large collection of sera has resulted. Some of these animals and sera have been supplied to biologists at the University of California, California Polytechnic College, University of Minnesota, Michigan State University, University of Florida, and Princeton University for various studies. Skin biopsies of a number of local mammals were also sent to workers at Dartmouth Medical School for chromosome studies. In addition, skulls, skins, and formalin specimens of rare Panamanian mammals have been deposited in the U.S. National Museum, Washington, D.C.

Significance to Bio-Medical Research and the Program of the Institute:

Because of its economic importance and its resemblance to foot-and-mouth disease, vesicular stomatitis has long been of interest to veterinary and other animal health agencies. The disease occurs in sporadic epizootics in the United States, but is enzootic in many areas of Latin America. Until recently, the disease was thought to be limited to the New World, but recent isolations of a new VSV serotype (Chandipura virus) from India and Nigeria suggest that this group of viruses may have a much wider distribution. Reports of human cases of vesicular stomatitis in the United States and results of immunologic surveys in Central America and Panamá indicate that the Indiana and New Jersey strains of VSV also infect man and may be of considerable public health importance. Reported clinical cases of the disease suggest that the virus produces an influenza-like illness in humans. At present, however, little is known about the natural history of the disease. Isolations of VSV-Indiana and Chandipura virus from naturally infected phlebotomine sandflies plus recent studies demonstrating VSV-Indiana transmission by experimentally infected sandflies suggest that these insects may serve as vectors of at least two VSV types. This project is designed to elucidate the ecology of VSV and the epidemiology of the disease. Related entomologic studies are providing considerable new information about the feeding patterns, vector potential and ecology of New World phlebotomine sandflies.

In addition to the economic and public health importance of VSV, studies of its ecology may yield new insights into the biology of some other bullet-shaped viruses. Morphologically, VSV is related to a diverse group of viruses which infect vertebrates (rabies is the most important), insects and plants. A number of these viruses are arthropod-transmitted, and three of them, including VSV, have been shown to be transovarially transmitted in insects. Although purely speculative, the possible implications are exciting!

Proposed Course of the Project:

Upon completion of the mark-and-release program, we plan to curtail our field work and devote most of our time to laboratory studies of sandflies and their associated viruses. The mechanism and species range of VSV transovarial transmission will be studied in detail, for this is our first clue of how these insects might acquire the virus in nature. Work will continue to develop breeding colonies of sandflies in the laboratory. In addition, attempts will be made to identify and characterize the non-VSV sandfly virus isolates. We also plan to study experimental sandfly infection and possible transovarial transmission with certain members of the Phlebotomus Fever group of arboviruses.

Publications:

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Tesh, R. B., Chaniotis, B. N., Carrera, B., and Johnson, K. M.: Further studies of the natural host preferences of Panamanian phlebotomine sandflies. _Amer. J. Epidemiol._, 1971 (in press).

Tesh, R. B., and Johnson, K. M.: Vesicular stomatitis. In Hubbert, W.T., McCulloch, W. F., and Schurrenburger, P. R. (Eds.): _Diseases Transmitted from Animals to Man_, Sixth Ed., Charles C. Thomas, Springfield, Ill.

LABORATORY OF MICROBIAL IMMUNITY

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PHS-NIH
Summary Statement
Laboratory of Microbial Immunity
July 1, 1970 through June 30, 1971

The major objective of the Laboratory remains the elucidation of basic mechanisms involved in cellular and humoral immune responses to microbial and tissue antigens. Germfree animals are used in experiments in which it is necessary to minimize prior exposure to naturally occurring antigens, or in which low rates of immunoglobulin synthesis before experimental manipulation are desirable; a special facility is maintained for work with germfree animals. Dr. Robert E. Tigelaar, a Research Associate, joined the Laboratory this year, and has been studying induction of graft-*versus*-host reactions. Dr. Philip R. B. McMaster was assigned to an administrative task force for the first four months of 1971. There have been no other changes in personnel. The importance of interaction among different kinds of lymphoid cell has been elucidated using graft-*versus*-host reactions as a model of cellular immune responses, and the antibody response to Type III pneumococcal polysaccharide as a model of humoral responses. Progress has been made in studies of the control of immunoglobulin synthesis, of the genetic and antigenic requirements for the production of immunity to protozoal parasites, and of the interaction of sensitized lymphoid cells and antibody in the production of immune tissue injury.

RESEARCH ACCOMPLISHMENTS

ANTITHYROID ANTIBODY INFLUENCES COURSE OF ALLERGIC THYROIDITIS

Allergic thyroiditis, a disease resembling certain forms of human thyroiditis, is produced in guinea pigs by immunization with extracts of thyroid emulsified in Freund's complete adjuvant. It is known that the disease can be produced in unimmunized guinea pigs by transfer of lymphoid cells, but not of serum, from immunized syngeneic donors. Strain 13 guinea pigs were immunized with guinea pig thyroid extract. Six to eight days later pools of their lymph node cells were transferred to syngeneic recipients, which then received rabbit anti-guinea pig thyroid antibody intraperitoneally. Recipients were sacrificed 12 days later, and their thyroids examined. Sensitized lymph node cells in combination with antibody produced more frequent and severe lesions in the thyroid than did cells alone. Antibody alone did not produce mononuclear infiltrates. Repeated injection of antibody had a greater effect than injection once. A single injection of antibody was more effective if given shortly after cells than if given 10 days after the cells. This study shows that the severity of allergic thyroiditis can be increased by antithyroid antibody, which produces no disease by itself. The combination of antibody with cells provides a model for the study of the contribution of each to autoimmune tissue destruction. (Dr. P. R. B. McMaster, Mr. M. B. Caldwell with Dr. G. Sharp (Dept. of Medicine, University of Columbia School of Medicine, Columbia, Mo.))

TRYPANOSOME VACCINE PROTECTS MICE

A major problem in the study of immunity to protozoal parasites concerns the difficulty of producing resistance to infection with killed vaccines, although some resistance is acquired following natural infection. Balb/c mice infected with _Trypanosoma rhodesiense_ (Wellcome strain) invariably succumb within five days. _T. rhodesiense_ was obtained by differential centrifugation of blood from infected mice. All mice given at least two injections either of heat-killed or frozen-thawed organisms were resistant to subsequent infection with this parasite. Mice immunized with killed crithidia of _T. rhodesiense_ obtained from _in vitro_ cultures were not resistant to infection. Immunization either with killed blood parasites or with crithidia resulted in the formation of antibody to both. This work provides a model for the study of immunity to parasites. Since inbred mice are used, requirements for cells, plasma proteins, antibodies, or combinations of these can be examined. (Dr. J. F. Finerty and Mr. C. B. Evans)

CELL PROLIFERATION AND DIFFERENTIATION IN ANTIBODY RESPONSE STUDIED SEPARATELY

Differentiation of antibody-forming cells from precursors and rapid proliferation of these cells occur simultaneously in immune responses to most antigens. Hydroxyurea or mitomycin C, two drugs which are known to inhibit cell division and to kill dividing cells were given to mice at various times after immunization either with sheep erythrocytes (SRC) or Type III pneumococcal polysaccharide (SSS-III). The numbers of antibody-forming cells were determined five days after administration of antigen. Almost no antibody-forming cells were found five days after immunization in animals given a single injection of either drug on days 1, 2, 3 or 4 following immunization with SRC. By contrast, these drugs produced significant reduction in antibody-forming cells to SSS-III only if given on days 1 or 2 after immunization; they are without effect if administered at later times. There is a rapid increase in the rate of antibody synthesis/cell between three and five days after immunization with SSS-III. These findings indicate that cell division and differentiation occur at different times after immunization with SSS-III, providing a unique opportunity to study one process in the absence of the other. (Dr. P. J. Baker, Mr. P. W. Stashak and Miss D. F. Amsbaugh with Dr. B. Prescott (Laboratory of Microbiology, NIAID))

TWO CELL TYPES REGULATE ANTIBODY RESPONSE

Treatment with antilymphocyte serum (ALS) has been shown to depress almost completely the immune response to a number of antigens. In contrast, the administration of ALS to mice immunized with Type III pneumococcal polysaccharide (SSS-III) leads to a 10-fold increase in the number of antibody-producing or plaque-forming cells (PFC) obtained. Since ALS exerts its effects primarily by acting upon thymic-derived lymphocytes, various numbers of thymus cells and peripheral white blood cells were given to ALS-treated mice in order to assess the influence of such cells on the magnitude of the PFC response. Thymus cells reduced the number of SSS-III-specific PFC obtained; the infusion of peripheral white blood cells resulted in a further increase in PFC. These effects were dependent on the dose of cells. The findings suggest that two functionally distinct types of cells (a suppressor cell and an amplifier cell) act in an opposing manner to regulate the antibody response to SSS-III. The ability of ALS to increase the magnitude of the antibody response to SSS-III is apparently the result of the inactivation of a type of cell that normally suppresses the antibody response produced following immunization. It is possible that this may be a general homeostatic control mechanism for regulating the rate of antibody synthesis by antibody-forming cells. (Dr. P. J. Baker, Dr. B. Prescott (Laboratory of Microbiology, NIAID), Mr. P. W. Stashak and Miss D. F. Amsbaugh)

LYMPHOID CELLS INTERACT IN LETHAL GRAFT-VERSUS-HOST REACTIONS

Graft-*versus*-host (GVH) reactions are produced when allogeneic cells are inoculated into hosts which are incapable of rejecting the cells. Such reactions are the principal impedimant to the transplantation of bone marrow or lymphoid cells in man for the correction of immunologic or hematopoietic disorders. The lethal potential of parental thymocytes, blood lymphocytes, or combinations of these cells was assayed in lightly irradiated newborn F_1 mice. The minimum number of cells causing significant (20-30%) mortality was 2.5×10^5 for PBL and 10^7 for thymocytes, respectively. A combination 10^5 PBL and 10^7 thymocytes killed almost all recipients. Large numbers of PBL (10^6) or thymocytes (2.5×10^7) were needed to kill all recipients. Death produced by thymocytes occurred later than that produced by PBL. The combination produced late mortality, similar to that found with large numbers of thymus cells. This study shows that lymphoid cells cooperate in the production of lethal GVH reactions. The data also indicate that in the combination one population of cells (the thymocytes) act as the precursors of cytotoxic lymphocytes; its activity is amplified by the other population (the blood lymphocytes). (Drs. R. E. Tigelaar and R. Asofsky)

ANTI-IMMUNOGLOBULIN SUPPRESSES IMMUNOGLOBULIN SYNTHESIS

Germfree Balb/c mice were treated from birth either with specifically purified goat anti-mouse IgM or goat γ-globulin, 1 mg/mouse/week. Mice that received goat γ-globulin had high levels of IgM and $7S\gamma 1$, and moderate levels of $7S\gamma 2$ and IgA. These mice produced antibody after intraperitoneal challenge with horse ferritin; in addition their sera contained antibody to at least one goat plasma protein and to sheep erythrocytes. By contrast, mice treated with anti-IgM had no circulating IgM, $7S\gamma 2$, or IgA, and low levels of $7S\gamma_1$. These mice did not produce antibody after challenge with ferritin, nor did their sera contain antibodies to goat proteins or sheep erythrocytes. Histologically, lymphoid tissues from animals that received goat γ-globulin showed follicular hypertrophy and contained many plasma cells; the numbers of plasma cells containing specific immunoglobulins as judged by immunofluorescence agreed well with the relative serum levels of these proteins. Mice that received anti-IgM had few plasma cells and almost no germinal centers. These experiments show that anti-IgM can interfere with the normal synthesis of immunoglobulins and antibody. The data suggest that IgM molecules, either associated with membranes or free in the extracellular fluid, are essential for the induction of normal humoral immune responses. (Dr. R. Asofsky and Miss M. B. Hylton with Drs. A. Lawton, III, M. D. Cooper and P. Kincaid (University of Alabama School of Medicine, Birmingham, Ala.))

Serial No. NIAID - 110
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Administration, research planning and coordination

Previous Serial Number: Same

Principal Investigator: John E. Tobie

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	24/12			24/12
Professional:	12/12			12/12
Other:	12/12			12/12

Project Description:

Administrative and supervisory support is provided to all personnel and to each project of the Laboratory.

Research plans are discussed with the various investigators, over-all evaluations made and the research activities of the Laboratory coordinated. Collaborative research with specialists in other laboratories of NIAID, or institutes of the NIH, or various university investigators is encouraged and assistance given the professional staff in effecting the necessary arrangements. Technical consultation is provided to individuals of the NIH, and to scientists of other departments of the Government or academic organizations. Assistance is provided the professional staff in the preparation of manuscripts and in editing and reviewing a variety of technical reports. The needs of all personnel are evaluated and an effort made to obtain the necessary equipment and supplies for the efficient conduct of research. Budget requirements are forecast on a yearly basis and emergency needs dealt with as problems arise. Supervision is given in regard to laboratory expenditures, travel of personnel and correspondence. Highly qualified professionals and technicians are sought as staff additions or replacements to insure a high level of research excellence.

Serial No. NIAID - 111
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The infectivity and pathogenicity of axenically cultivated strains of Entamoeba histolytica, with intracellular virus particles, for germfree and conventional experimental animals; the pathogenesis of experimental amoebic enteritis

Previous Serial Number: Same

Principal Investigator: Bruce P. Phillips

Other Investigators: Louis S. Diamond* (Project Subtitle I)
Akio Takeuchi** (Project Subtitle II)

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Md.
**Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	5/12			5/12
Professional:	3/12			3/12
Other:	2/12			2/12

Project Description:

Project Subtitle I: Infectivity and pathogenicity of axenically cultivated strains of E. histolytica, with intracellular virus particles, for germfree and conventional experimental animals

Objectives:

Previous attempts to produce amoebic enteritis by inoculation with axenically cultivated amoebae have been unsuccessful. It is of interest to ascertain whether such amoebae, when harboring virus, may produce disease.

Methods Employed:

E. histolytica containing intracellular virus are derived from axenic cultures and inoculated intracecally into germfree and conventional guinea pigs. The animals are sacrificed at appropriate intervals and examined for the presence of amoebae and amoebic lesions.

Major Findings:

Virus-laden *E. histolytica* from axenic culture have failed to produce either lesions or infections in germfree and conventional guinea pigs.

Significance to Bio-Medical Research and the Program of the Institute:

It is important to ascertain whether associated virus may influence the pathogenicity of the amoeba or the pathogenesis of amoebic disease. Thus far there is no evidence to support any viral influence but considerably more investigation is required for valid conclusions in this regard.

Proposed Course of Project:

To investigate other virus-laden strains of the amoeba, particularly those that have been in axenic culture for only short periods of time. Also, to harvest virus particles from freeze-disrupted amoebic cells and add these to amoebic inocula that normally produce lesions in experimental animals. This may indicate whether the associated virus has any influence on the amoeba or the development of amoebic disease.

Project Subtitle II: The pathogenesis of experimental amoebic enteritis

Objectives:

To ascertain how *E. histolytica* gets from the intestinal lumen into the tissues.

Methods Employed:

Germfree and controlled flora guinea pigs are inoculated intracecally with amoeba from known virulent cultures and sacrificed at various intervals of a few hours up to several weeks. Enteric tissues are collected and appropriately prepared for observation by light and electron microscopy. Numerous electron micrographs are prepared and searched for amoebae in the various stages of tissue penetration from mere contact with the microvillae to invasion of the serosa.

Major Findings:

Several hundred electron micrographs of the amoebae in various areas of intestinal tissue have been prepared and are being studied. The interpretation of these EM observations is a very complex problem and delineation of major findings must await completion of detailed study of these and possibly additional electron micrographs.

Significance to Bio-Medical Research and the Program of the Institute:

The pathogenic mechanisms in amoebic enteritis have long been the subject of considerable speculation and differing opinions, none of which can be scientifically supported. These studies show promise of providing some elucidation of this scientific enigma.

Proposed Course of Project:

The studies are nearing completion and the observations will be presented in manuscript form for publication and possible presentation at the next annual meeting of the American Society of Tropical Medicine and Hygiene.

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-A
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genesis of L-histidine decarboxylose in the gastric and duodenal mucosa

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Charles H. Zierdt*
Zdenka Horakova**
Michael A. Beaven**

Cooperating Units: *Laboratory of Clinical Pathology, CC, NIH, Bethesda, Md.
**Experimental Therapeutics Branch, NHLI, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	3/12			3/12
Other:	1/12			1/12

Project Description:

Objectives:

To ascertain the origin of this common enzyme of the upper gastrointestinal tract.

Methods Employed:

The abundant presence of this enzyme in man and the common laboratory animals as well as in cultures of Lactobacilli was previously confirmed. Samples were taken from germfree Sprague-Dawley rats and from ex-germfree rats of this strain that were monocontaminated with a lactobacillus isolated from man. The samples were assayed by standard methods for the enzyme of above reference.

Major Findings:

L-histidine decarboxylase was not present in the gastric and duodenal scrapings and contents of germfree rats. The enzyme was present, however, in rats harboring only a lactobacillus. The lactobacillus exhibited a very high rate of growth in the stomach of the ex-germfree animals and formed therein a pseudo-membrane of bacteria just as in the conventional host.

Significance to Bio-Medical Research and the Program of the Institute:

The results tend to refute the popular and long-held theory that L-histidine decarboxylase is of mammalia origin. The data suggest that, at least in the rat, the enzyme is of microbial origin and the prodigious growth of *Lactobacillus* that occurs in the stomach of man and animal is a possible and, indeed, likely source.

Proposed Course of Project:

Completed

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-B
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Production of specific antisera to purified human gamma globulin

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Helmut Brunner*

Cooperating Units: *Laboratory of Infectious Diseases, NIAID, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	3/12			3/12
Other:	1/12			1/12

Project Description:

Objectives:

To produce antisera to human gamma globulin, free from antibodies, to Mycoplasma sp., for use in radioimmunodiffusion test for mycoplasma in humans.

Methods Employed:

Germfree guinea pigs are inoculated intradermally with purified human gamma globulin in complete Freund's adjuvant followed by similar inoculations in three and six weeks, respectively, with the purified globulin in incomplete Freund's adjuvant. The animals are sacrificed and bled five days following the final inoculation and the sera is collected and pooled.

Major Findings:

The sera from all conventional animals comprising the many species available at NIH had a serum fraction that cross-reacted with the mycoplasma antigen used in screening human sera for antibodies to mycoplasma. The sera from germfree guinea pigs has been free of antibody to mycoplasma and has shown a high titer to human gamma globulin following the aforementioned inoculations.

Significance to Bio-Medical Research and the Program of the Institute:

The studies have provided an essential ingredient to the radioimmunodiffusion test for mycoplasma. This more sensitive test indicates the number of humans with antibodies to mycoplasma approaches 90 percent instead of the 40-50 percent figure arrived at with previous, less sensitive tests.

Proposed Course of Project:

The project will be completed when sufficient antisera is produced to facilitate the screening program which is contemplated--possibly in about one month.

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-C
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biologic investigation of Blastocystis hominis in germfree and controlled flora experimental hosts

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Charles H. Zierdt*

Cooperating Units: *Laboratory of Clinical Pathology, CC, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	3/12			3/12
Other:	1/12			1/12

Project Description:

Objectives:

To evaluate the recently published disclosure that B. hominis, long considered a harmless yeast, may be a protozoan with several stages in its life cycle including an ameba form.

Methods Employed:

Blastocystis hominis, isolated from patients in the Clinical Center, is maintained in culture with a mixed bacterial flora. The flora is thus far essential to maintenance of the Blastocystis in vitro. Drug sensitivity studies indicate methods of controlling the flora, thus permitting the preparation of a bacteria-free inoculum of B. hominis for introduction into germfree and controlled flora animals. Animals inoculated via various routes are examined for presence of the several described forms of the organism in the lumen and in the tissues.

Major Findings:

The treatment of a cultured inoculum of B. hominis with a preparation comprising eight antibiotics has effectively inhibited bacterial growth without adversely affecting the Blastocystis. This has permitted the intracecal, oral, and intraperitoneal inoculation of germfree guinea pigs with B. hominis without actively growing bacteria. Animals that received a subsequent injection of the combined antibiotics on post-inoculation day three remained free from actively growing bacteria for about eight days. Although rare B. hominis were observed in cecal samples of intracecally inoculated animals sacrificed up to the fifth post-inoculation day, these were believed to be "stragglers," from the inoculum, and infection apparently did not occur. This may be due to the comparatively high redox potential of the germfree guinea pig cecum. No B. hominis were recovered from animals inoculated orally or intraperitoneally.

Significance to Bio-Medical Research and the Program of the Institute:

If animals can be infected with B. hominis derived from a human source, accompanying observations may tend either to confirm or deny that this microorganism should be reclassified as a protozoan. Furthermore, if it should indeed be a protozoan, it is important to determine if it possesses any pathogenic potential either alone or synergistically.

Proposed Course of Project:

To continue the attempt to infect experimental animals by addition of one or more known species of viable bacteria to the inoculum.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The genetic, cellular and regulatory factors in autoallergic disease

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: Michael Kyriakos*
Gordon Sharp**
William Irwin**
Vernon Wong***

Cooperating Units: *Department of Pathology, Barnes Hospital, St. Louis, Mo.
**Department of Medicine, University of Columbia Medical School, Columbia, Mo.
***National Eye Institute, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	22/12			22/12
Professional:	3/12			3/12
Other:	19/12			19/12

Project Description:

Project Subtitle I: Effect of antibody on the development of autoimmune disease

Objectives:

To determine the influence of antibody upon the development of autoimmune disease using the cell transfer system.

Methods Employed:

Guinea pigs were immunized with guinea pig thyroid extract emulsified in Freund's complete adjuvant. Six to eight days later the lymph node cells from the draining lymph nodes were harvested, washed and injected intraperitoneally into histocompatible recipients. Rabbit or guinea pig anti-thyroid antibody was then injected intraperitoneally every other day at selected intervals. At the end of the selected interval (12 days) the thyroid glands were removed, fixed in formalin, sectioned, and stained with hematoxylin and eosin. These were then examined to determine the frequency and severity of thyroiditis. The thyroiditis was classified as acute (esinophilic infiltrate) or chronic (mononuclear cell infiltrate).

Major Findings:

The rabbit antibody alone was capable of producing a transient esinophilia in the thyroid. Preliminary study suggested that this antibody also enhanced the effect of cell transfers. Ordinarily, lymphoid cell transfers produced a minimal level of thyroiditis in only 30% of the recipients. The rabbit antibody, however, increased the frequency of chronic thyroiditis in recipients to 100% when given every other day. These experiments have been repeated and confirmed. The antibody also increased the intensity of the chronic thyroiditis, so that many of the animals had a more severe disease than would have developed in the donors.

The rabbit antibody can be somewhat effective if given once shortly after the cells are transferred, but is less effective when given once than when given repeatedly. It appeared to be more effective when given shortly after the cells than when given 10 days after the cells.

Significance to Bio-Medical Research and the Program of the Institute:

The combination of antibody with sensitized cells markedly altered the effect of the cells and may provide a system with which to analyse the role of each in the production of autoallergic tissue destruction.

Proposed Course of Project:

To define the amount of antibody and number of cells needed and to determine the nature of each. To determine the effect of this antibody upon the different strains of guinea pigs.

Project Subtitle II: Effect of metabolic inhibitors on the transfer of allergic thyroiditis

Objectives:

To measure the effect of metabolic inhibitors upon the development of chronic allergic thyroiditis induced by the transfer of lymphoid cells from immunized donors to histocompatible recipients. This is done to determine whether the cells transferred influence the cells of the recipients through some metabolic path other than that of active immunization.

Methods Employed:

The methods employed are those previously given plus the injection of methotrexate (MTX) intraperitoneally (IP) at suitable intervals.

Major Findings:

MTX, given twice a week in doses of 5 mg/100 gm body weight, prevents the development of chronic allergic thyroiditis if the drug treatment is initiated shortly after active immunization. If given to recipients of lymphoid cells that were obtained from donors immunized with thyroid, then it also appears to be effective. However, if it is given only once, shortly after the cell transfers, it is not effective.

Significance to Bio-Medical Research and the Program of the Institute:

Methotrexate can prevent the development of thyroiditis by active immunization if given twice a week from the time of immunization. However, if given after the immunization has been effective, the MTX does not seem to be effective. In the cell transfer system MTX appears capable of inhibiting the development of chronic thyroiditis. This implies that to cause thyroiditis, the transferred cells must be capable of multiplying or of influencing other cells that multiply.

Proposed Course of Project:

To repeat the experiments to confirm the results. To determine the nature of the cells that are involved in the formation of the disease.

Project Subtitle III: Relation of macrophage migration inhibition factors (MIF) to the induction of thyroiditis

Objectives:

To determine the relation of MIF to the development of experimental allergic thyroiditis in guinea pigs.

Methods Employed:

Selected quantities of thyroid extract were emulsified in Freund's adjuvant. Guinea pigs were immunized with one dose and examined 6 weeks later as described previously.

Major Findings:

Amounts of thyroid extract in the immunizing emulsion that were too small to produce detectable thyroiditis or detectable delayed skin hypersensitivity, did alter the animals leukocytes so that they produced MIF upon contact with thyroid extract.

Significance to Bio-Medical Research and the Program of the Institute:

The work suggests that sensitization of cells so that they can produce MIF in response to exposure to an auto antigen is inadequate to produce disease. If MIF is involved in the disease process, it seems likely it acts in conjunction with one or more other factors.

Proposed Course of Project:

To repeat the study.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113-C
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Allergic ocular inflammation

Previous Serial Number: None

Principal Investigator: Philip R. B. McMaster

Other Investigators: Samuel B. Aronson*
Thomas Moore*

Cooperating Units: *Department of Ophthalmology, University of California School of Medicine, San Francisco, Calif.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To determine the role of cells and serum of immunized animals upon the eyes of animals immunized with retina extract.

Methods Employed:

Guinea pig retinal extract emulsified in Freund's adjuvant is used to immunize guinea pigs. Their lymph nodes and spleen leukocytes are transferred to recipients and their serum given to others. The eyes are examined with the slit lamp and then sectioned for histologic analysis.

Major Findings:

The leukocytes from immunized donor guinea pigs produced an allergic uveitis when put into recipient animals. The serum did not do so. When the serum globulins were labelled with iodine, they did not show any affinity for the eye when injected intravenously or intravitreously.

Significance to Bio-Medical Research and the Program of the Institute:

The work suggests that cellular allergy of some sort is a primary cause of experimental autoallergic uveitis.

Proposed Course of Project: Terminated

Honors and Awards: None

Publications: None

Serial No. NIAID - 113-D
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of lincomycin derivatives upon acute toxoplasmosis of mice

Previous Serial Number: None

Principal Investigator: Philip R. B. McMaster

Other Investigators: John F. Finerty, LMI, NIAID, NIH, Bethesda, Md.
Kendall Powers*
Milford N. Lunde*

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	2/12			2/12
Professional:	1/12			1/12
Other:	1/12			1/12

Project Description:

Objectives:

To improve the therapy of toxoplasmosis in man.

Methods Employed:

Mice are inoculated with virulent toxoplasma organisms. Some are then fed daily selected doses of lincomycin or V24 (another derivative of lincomycin) for 14 days. Depending upon the experiment, the treatment is started before the inoculation of organisms, at the time of inoculation or at a later date.

Major Findings:

Lincomycin and V24 protect almost all the mice when given before or at the time of infection. When given a day after infection they protect most mice. When given 3 days after infection they protect about half, enabling them to survive. Without treatment the infected controls die in 7 days. V24 seems the more effective. It not only keeps the animals clinically well, but also may prevent the development of the subclinical chronic cerebral form of the disease.

Significance to Bio-Medical Research and the Program of the Institute:

Currently sulfonamides and Dacaprim are used in the treatment of both the acute form and the recurrent chronic form of Toxoplasmosis. Patients being treated for recurrent disease often develop sensitivities to the sulfonamides, making an alternative very useful. Since the current therapy does not prevent the development of the chronic form of the disease, a drug that could do so would be most useful, particularly to prevent the development of that disease in adults, in the foetus and to cure the ocular disease form.

Proposed Course of Project:

To confirm the findings and to study the effect of the drugs on the cystic form and the recurrence of ocular disease.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunologic Tolerance

Previous Serial Number: None

Principal Investigator: Philip R. B. McMaster

Other Investigators: John F. Finerty, LMI, NIAID, NIH, Bethesda, Md.
Kendall Powers*
Arthur Schade**
Benjamin Prescott**
William R. Carroll***
Gerald Schiffmann****

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Md.
**Laboratory of Microbiology, NIAID, NIH, Bethesda, Md.
***Laboratory of Biophysical Chemistry, NIAMD, NIH, Bethesda, Md.
****Downstate Medical School, Brooklyn, N.Y.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Project Subtitle I: The relation of primary and secondary molecular structure to the induction of tolerance

Objectives:

To determine the minimal molecular size that will induce immunity and immunological paralysis and to determine whether the paralysis is dependent totally upon the primary chemical structure or whether the molecular length is also an important factor.

Methods Employed:

Pneumococcal polysaccharide Type III is broken by acid hydrolysis or sonication into small pieces which are then separated according to size on Sephadex G200 superfine columns. Selected doses are injected into mice, which are bled at appropriate intervals. Then the mice are reinjected with the same or another preparation of polysaccharide and bled again at suitable intervals.

Major Findings:

Our early work showed that polysaccharide with a molecular weight of 60,000 could immunize and paralyze, as could a smaller fragment with a weight of 27,000. A much smaller preparation which was very heterogenous and had a smaller molecular weight, was inactive. Further effort, however, yielded a preparation with an average molecular weight of 14,000. This immunized in very high doses suggesting the bulk of the material was inactive. However, when tolerance was produced with material with a molecular weight of 27,000, the mice did not make antibody when challenged with material with a molecular weight of 60,000.

Significance to Bio-Medical Research and the Program of the Institute:

A better understanding of specific immune suppression, of which pneumococcal paralysis is an example, may aid in the therapy of disease such as systemic lupus erythematosus and rheumatoid arthritis.

Proposed Course of Project:

This work began in 1959 with the preparation of the purified products. It was deferred, during an absence of several years. In 1964 it was recommenced with pilot experiments, designed with the advice of Drs. Kabat, Heidelberger and Gobel, to determine the possibility of a variety of experiments that might clarify the biophysics and biochemistry of tolerance. The preliminary definition of molecular structure requirements has now been established. Very preliminary work indicates that tolerance can easily be produced in vitro. The stage is then set to pursue these subjects to determine whether tolerance is primarily a question of the number of molecules versus the number of target cells, or the number of antigenic groups versus the number of target cells, and to study the metabolic paths required as outlined in our annual report of 1966.

Project Subtitle II: The production of tolerance in primates

Objectives:

To determine whether immune paralysis can be induced in a species other than a mouse--in this case a primate.

Methods Employed:

Pneumococcal polysaccharide Type III molecular weight 60,000 is injected intramuscularly into monkeys. They are bled weekly. The serum antibody is measured by the Farr test, and by agglutination tests.

Major Findings:

One to 50^{u}grams of the polysaccharide immunized the animals. Such animals, when given 10 ug of polysaccharide, no longer had detectable antibody. Upon reimmunization they did not produce detectable antibody. They retained the antigen in their serum.

Significance to Bio-Medical Research and the Program of the Institute:

The work shows that immunologic paralysis can be induced in a species other than the mouse, i.e. a primate. The matter is of importance to the design of dose schedules for immunization of man with this and similar antigens.

Proposed Course of Project:

The effect of larger antigens of the same primary chemical structure will be studied to determine the effect of molecular size in the immune response.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113-F
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Administration

Previous Serial Number: None

Principal Investigator: Philip R. B. McMaster

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	4/12			4/12
Other:	0/12			0/12

Project Description:

Objectives:

Assigned to Federal Assistance Streamlining Task Force to investigate the organization of the Division of International Studies in the Office of Education of HEW.

Serial No. NIAID - 116
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunoglobulin formation in germfree mice

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: Martha B. Hylton, LMI, NIAID, NIH, Bethesda, Md.
Harvey I. Cantor*
Michael Potter**
Elizabeth Bridges**
Alexander R. Lawton, III***
Max D. Cooper***
Paul Kincaid***

Cooperating Units: *National Institute for Medical Research, London, England
**Laboratory of Biology, NCI, NIH, Bethesda, Md.
***University of Alabama School of Medicine, Birmingham, Ala.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	35/12			35/12
Professional:	5/12			5/12
Other:	30/12			30/12

Project Description:

Objectives:

To study the response of germfree animals to a variety of immunologic stimuli with respect to levels in serum and relative rates of synthesis of various classes of immunoglobulins.

Methods Employed:

Immunoglobulin levels are measured by radial immunodiffusion using agarose plates impregnated with monospecific antisera. Biosynthesis is studied by incubating tissues in vitro in a medium containing C-14 labeled amino acids. Media are then tested for newly synthesized proteins by means of radioimmunoelectrophoresis. Antibodies are measured by passive hemagglutination, by use of specific immunoadsorbents, and by a complement-dependent bactericidal assay. Plasma cell tumors are induced by inoculation of light mineral oil intraperitoneally. Immunoglobulin synthesis is suppressed by treating mice from birth with goat anti-mouse IgM.

Major Findings:

Sera from unimmunized germfree mice contain only $7S\gamma_1$ and IgM. Immunization with ferritin or other proteins results in a transient increase in IgM and a sustained increase in $7S\gamma_1$. A second injection of the same (but not another) antigen results in a sustained increase in IgM levels, further increases in $7S\gamma 1$ and the first appearance of $7S\gamma_2$. Specific antibody accounts for less than 10% of the IgM, less than 20% of the $7S\gamma_1$, and less than 40% of the $7S\gamma_2$. Increasing the interval between the first and second injection of antigen results in increased antibody titers, with no increase in the fraction of each immunoglobulin that can be accounted for as specific antibody.

Intraperitoneal inoculation of an endotoxin results in a sustained increase in IgM with doses of antigen between 5 x 10^{-4} μg and 50 μg. IgM and bactericidal levels are well correlated, but less than 10% of the IgM is specific antibody. Inoculation of a "native protoplasmic polysaccharide" from *E. coli*, a material with molecular weight about 110,000, results in increases in IgM levels comparable to those seen with endotoxins.

Germfree Balb/c mice inoculated with light mineral oil develop very few plasma cell tumors in contrast to the high incidence obtained in conventional animals, as reported by McIntire and Princler. Germfree Balb/c mice monocontaminated by feeding *Salmonella Tel-Aviv* then inoculated with mineral oil develo plasma cell tumors in much higher incidence than do uncontaminated mice, but in lower incidence and much later than conventional mice. All of the tumors produced by monoinfected germfree mice that have been examined thus far produced IgA myeloma proteins. Mineral oil granulomas synthesized small amounts of all classes of immunoglobulins within one week of inoculation.

Lactating mammary gland from germfree mice secretes no detectable IgA, but produces *in vitro* a protein of low molecular weight (about 4S) that coprecipitates with IgA. Incubation of the gland with a 7S IgA myeloma protein results in a shift of this radioactive protein to the 11S fraction. No shift in the 7S IgA protein itself is seen. Preliminary studies suggest binding is more efficient to polymer IgA myeloma proteins.

Treatment of germfree mice from birth with goat anti-IgM results in reduction or elimination of all immunoglobulins, especially IgM, in the circulation. Mice treated with goat γ-globulin have elevated immunoglobulin levels and circulating antibodies to goat serum proteins; mice treated with anti-IgM lack such antibodies, but have residual heterologous anti-IgM in the circulation.

Significance to Bio-Medical Research and the Program of the Institute:

Most immunoglobulin synthesis in the immunized germfree animal is a result of experimental manipulation. Stimulated synthesis of immunoglobulins, especially of IgM, does not represent antibody synthesis. The increase with time in the fraction of the increased immunoglobulins that are specific antibody is reminiscent of the increases of affinity of antihapten antibodies with time, and probably represents a continued selection of "more specific" cells.

It is likely that the protein synthesized by mammary tissue is a homologue of "T-chain." This is the only example of T-chain synthesis in experimental animals occurring in the absence of IgA synthesis, and affords an opportunity for the study of the control of synthesis of this protein and of the assembly of secretory IgA molecules.

Antigenic stimulation via the gut seems to be important for the development of plasma cell tumors. Increasing numbers of plasma cell tumors have been shown to have defined antibody activity. Studies of plasma cell neoplasia with limited antigenic stimuli should help in the understanding of the pathogenesis of this type of tumor. Mice treated with anti-IgM show profound hypogammaglobulinemia. The condition produced by this type of treatment resembles certain kinds of human agammaglobulineamia, and may serve as a model for the study of this disease.

Proposed Course of Project:

An adaptive transfer system together with cell fractionation procedures (116-G and 116-I) will be employed to examine the origin and fate of cells synthesizing $7S\gamma 2$ immunoglobulins in secondary responses to proteins. The *in vitro* biosynthetic studies will be put on a quantitative basis using coprecipitation and liquid scintillation counting. The capacity of the putative T-chain to bind to a variety of IgA proteins of different size and structure will be studied in more detail. Plasma cell tumor induction will be attempted in germfree mice fed endotoxins, protein antigens, or killed bacteria. Animals treated with anti-IgM will be immunized with a variety of antigens. Germfree mice will be treated with anti-IgG and anti-IgA.

Honors and Awards: None

Publications:

Biozzi, G., Asofsky, R., Lieberman, R., Stiffel, C., Mouton, D. and Benacerraf, B.: Serum concentrations and allotypes of immunoglobulins in two lines of mice selected for "high" or "low" antibody synthesis. *J. Exp. Med.*, *132*: 752, 1970.

Serial No. NIAID - 116-G
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Fractionation of lymphoid cells

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: Robert E. Tigelaar, Phillip J. Baker, John F. Finerty and Philip R. B. McMaster, LMI, NIAID, NIH, Bethesda, Md.
Harvey I. Cantor*
Mark A. Mandel**
Carl Pierce***
John D. Stobo***
William E. Paul***

Cooperating Units: *National Institute for Medical Research, London, England
**Department of Surgery, University Hospitals of Cleveland, Cleveland, Ohio
***Laboratory of Immunology, NIAID, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	16/12			16/12
Professional:	4/12			4/12
Other:	12/12			12/12

Project Description:

Objectives:

To explore methods of fractionating lymphoid cells, so that cells mediating various immunologic reactions can be concentrated, and possibly completely purified.

Methods Employed:

Cells from solid lymphoid tissues in the form of suspensions, from blood or thoracic duct lymph, and from peritoneal exudates are centrifuged at high speed (10,000 x G) in gradients of bovine serum albumin. Fractions are collected with a peristaltic pump fractionator. Immunoglobulin synthesis is studied by incubating aliquots from each fraction in a medium containing C-14 labeled amino acids. Newly synthesized proteins are then identified by radioimmunoelectrophoresis. Localized hemolysis-in-gel is used to identify cells synthesizing anti-erythrocyte antibodies. Graft-*versus*-host activity is determined by inoculating cell fractions into newborn allogeneic recipients and determining the degree of splenomegaly in recipients nine days later. Fractions containing cells mediating delayed hypersensitivity reactions are identified by their capacity, when exposed to antigen, to inhibit migration of macrophages from capillary tubes. Cells carrying the capacity to mediate secondary responses are identified by transferring fractions to lethally irradiated syngeneic recipients, challenging with antigen, and measuring peak antibody titers by passive hemagglutination 7-10 days later. Immune responses to sheep erythrocytes are studied by incubating fractions *in vitro* with antigen and assaying for antibody-forming cells by local hemolysis-in-gel.

Major Findings:

Cells distribute in the gradient according to their buoyant density. Equilibrium is achieved after 30 minutes at 10,000 x G. Isolated bands are recovered in identical fractions upon recentrifugation. Recovery of cells from individual fractions is reproducible (± 10%) as is activity. Total cell recovery ranges from 60-80% and functional recovery exceeds 75%. Antibody and immunoglobulin-forming cells are found in fractions of intermediate density. Five to 20-fold concentrations of such cells have been obtained. Cells from spleen mediating graft-*versus*-host reactions are found in the most dense fractions. Five to 10-fold concentration factors have been obtained. Cells from thymus mediating graft-*versus*-host activity are found in the most dense and in the most buoyant fractions. Fractions of intermediate density show reduced activity. Only the most buoyant fractions from thymus are capable of producing *in vitro* mixed leukocyte reactions. Cells mediating *in vitro* delayed hypersensitivity reactions are recovered from similar fractions. Cells from spleen mediating adaptive transfer of immunologic memory are found in all fractions for the first 6-8 weeks after immunization, but display a more restricted distribution thereafter. Active fractions 12-20 weeks after immunization are those with intermediate density.

Cells capable of responding *in vitro* to sheep erythrocytes are distributed throughout the gradient, both before and after immunization. Peak activity is found in the most buoyant fractions.

Significance to Bio-Medical Research and the Program of the Institute:

This procedure permits precise and reproducible fractionation of different functional activities. Separation of immunological memory and immunoglobulin synthesis on one hand from cellular immune functions (graft-*versus*-host activity) on the other provides direct proof that these reactions are initiated by different groups of precursor cells. Dissociation of *in vivo* graft-*versus*-host activity and *in vitro* mixed leukocyte reactions suggests that more than one cell type participates in homograft reactions, and shows that the mixed leukocyte reaction may not predict potent *in vivo* reactions.

Evidence has been obtained that immunologic memory is a function that grows with time for several weeks, then becomes stable (see 116-I). The heterogeneous distribution of spleen cells at early times following priming is regarded as evidence for rapid cell turnover, while restriction of activity in thoracic duct cells is believed to be the result of the accumulation of a single non-dividing cell type.

Proposed Course of Project:

The buoyant density distribution of cells interacting synergistically in graft-*versus*-host reactions will be studied in populations obtained from blood, thymus, and spleen both before and after treatment with heterologous antithymocyte serum (see NIAID - 117 and 116-J). An attempt will be made to segregate "carrier reactive" from "hapten reactive" cells in adoptive transfer of secondary responses to hepten-protein conjugates.

Honors and Awards: None

Publications: None

Serial No. NIAID - 116-I
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of thoracic duct lymphocytes of mice

Previous Serial Number: NIAID - 118

Principal Investigator: Richard Asofsky

Other Investigators: Harvey I. Cantor*
Mark A. Mandel**
Nelson A. Wivel***

Cooperating Units: *National Institute for Medical Research, London, England
**Department of Surgery, University Hospitals of Cleveland, Cleveland, Ohio
***Viral Leukemia and Lymphoma Branch, NCI, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	5/12			5/12
Professional:	1/12			1/12
Other:	4/12			4/12

Project Description:

Objectives:

To study the distribution and function of cells in different parts of the lymphoid system.

Methods Employed:

Spleen or thoracic duct cells were obtained from Balb/c mice at various times following a single injection of ferritin. Aliquots of cells from each pool were transferred to lethally irradiated syngeneic recipients, which were then challenged with ferritin. Peak (7-10 day) antibody titers were measured by passive hemagglutination, and the classes of immunoglobulins with antibody activity determined by radioimmunoelectrophoresis. In most experiments, recipients of thoracic duct cells also received syngeneic bone marrow cells. Cells were fractionated on gradients of bovine serum albumin (BSA) (see 116-G).

Major Findings:

The capacity of spleen cells to transfer immunologic memory, as judged by peak titers in recipients of these cells remained stable for 8 weeks after priming, then rose somewhat to a peak at 12 weeks, and remained stable for a further 28 weeks. This function in thoracic duct cells rose exponentially for 8 weeks, with a doubling time of 10 days, and attained a much higher level than in spleen cells. Thereafter, thoracic duct activity fell to the level of spleen cells and remained stable for 28 weeks. Antibody formed in recipients both of spleen and thoracic duct cells was of $7S\gamma_1$ and $7S\gamma_2$ types exclusively. Thoracic duct drainage had no effect on the capacity of spleen cells to transfer immunologic memory.

Cells from spleen mediating adoptive transfer of immunologic memory were widely distributed after fractionation on gradients of BSA. This heterogeneity was observed for 8 weeks following primary immunization. Thereafter a more restricted distribution was observed, with peak activity in fractions of specific gravity 1·072-1·076. At all times after immunization the distribution of cells from thoracic duct mediating transfer of secondary responses showed a restricted distribution identical to that seen for spleen late after immunization.

Significance to Bio-Medical Research and the Program of the Institute:

With respect to immunologic memory the experiments indicate that the circulating lymphocytes and cells in solid lymphoid tissues represent two distinct pools of reactive cells. It seems likely that the former is responsible for dissemination of cells, while the latter are responsible for their generation. There is a reserve of all cell components needed for immunologic memory in the solid lymphoid tissues. Generation of new "memory lymphocytes" appears to continue for many weeks after priming with a soluble antigen.

Proposed Course of Project:

It will be determined whether one or both of the cell types participating in GVH reactions are removed by thoracic duct drainage. The effect of thoracic duct drainage on "carrier-reactive" cells in hapten-protein antibody responses will be examined.

Honors and Awards: None

Publications: None

Serial No. NIAID - 116-J
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The suppressive effects of heterologous antithymocyte sera on the homograft reactions in mice

Previous Serial Number: NIAID - 118-A

Principal Investigator: Richard Asofsky

Other Investigators: Robert E. Tigelaar, Laboratory of Microbial Immunity, NIAID, NIH, Bethesda, Maryland
Harvey I. Cantor*

Cooperating Units: *National Institute for Medical Research, London, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	10/12			10/12
Professional:	2/12			2/12
Other:	8/12			8/12

Project Description:

Objectives:

To investigate the mechanism of action of antithymocyte sera in suppressing graft-versus-host reactions and allograft rejection.

Methods Employed:

Antithymocyte sera (ATS) were prepared by immunization of rabbits with two intravenous injections of Balb/c thymus cells. Graft-versus-host assays are performed by inoculating spleen cells from mice into newborn allogeneic recipients, and measuring the resulting splenomegaly 9 days later. The activity of cells from animals receiving different doses of ATS was measured with a GVH assay 2 days after treatment. In addition, the capacity of blood lymphocytes (PBL) to potentiate the activity of normal thymus cells was measured (see NIAID - 117). In cases in which the dose of ATS was sufficient to suppress the activity of spleen cells, restoration of the activity of these cells by the addition of a small number of PBL was attempted. The effect of ATS on GVH reactivity is studied using a death assay (see NIAID - 117) as well. Finally, the effects of ATS on interacting cells from germfree (GF) mice are examined.

Major Findings:

Pretreatment of donors with 0.05 ml of ATS produces less than a 20% reduction in the GVH activity of PBL, but produces an 80-90% reduction in the capacity of these cells to interact synergistically with thymus cells. Treatment of donors with 0.25 ml of ATS reduces the GVH activity of spleen cells by more than 80% but these cells can be restored to almost normal reactivity by addition *in vitro* of 10^5 normal PBL. Treatment with 0.5 ml ATS produces a slightly greater decrease in activity of spleen cells, but these cells can be restored only slightly by the addition of normal PBL. Moderate doses of ATS reduce the activity of cells from treated GF mice measured directly; spleen cells from such mice are restored poorly by addition of normal PBL.

Significance to Bio-Medical Research and the Program of the Institute:

ATS is a potent and useful immunosuppressant which has found important clinical use. This study shows that 2 cell components required for GVH activity are differently susceptible to heterologous ATS; rather large doses of ATS are required to suppress both cell types. It is probable that more effective suppression can be obtained by combining ATS with treatment that affects primarily the cells least susceptible to ATS.

Proposed Course of Project:

The remarkably high susceptibility of germfree mice to ATS will be examined further for sensitivity of the individual cell components involved in synergy. The rates of recovery, from a large dose of ATS, of each of the interacting cell components in spleen and the circulation will be determined. The effects of ATS on the thymus will be tested for their capacity to suppress the cells that are relatively resistant to ATS.

Honors and Awards: None

Publications: None

Serial No. NIAID - 117
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The immunologic response of conventional and germfree mice to blood-induced infections of Plasmodium berghei

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID, NIH, Bethesda, Md.
Camille L. Hyde*

Cooperating Units: *Department of Biology, American University, Washington, D.C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	30/12			30/12
Professional:	6/12			6/12
Other:	24/12			24/12

Project Description:

Objectives:

To compare the parasitemia, immunoglobulins and serum antibody titers produced in mice infected with the less virulent 17X strain of Plasmodium berghei to those produced when mice are infected with the virulent NYU-2 strain. To determine whether survivors of the 17X infection are resistant to an NYU-2 challenge.

Methods Employed:

Sera were prepared daily from germfree (GF) and conventionally-reared (CV) mice infected with the 17X strain of P. berghei. Parasite counts are determined from stained blood smears. Immunoglobulin levels are measured by a radial gel diffusion technique using specific antisera. Serum antibody titers are assayed by the indirect fluorescent antibody technique.

Major Findings:

Germfree (GF) and conventionally-reared (CV) NIH-white mice infected with 17X showed a 90% survival rate compared to no survivors in mice infected with the NYU-2 strain. The 17X parasitemia revealed a 4-5 day leveling off during the first 10 days of infection, and the parasitemia became subpatent by day 20. This contrasted with the rapidly rising NYU-2 parasitemia which was accompanied by death of all mice. Serum antibody titers to 17X were detected on days 6 and 7 in GF and CV mice, respectively. The titers increased to a mean of 1:400 in both groups of mice. GF mice infected with NYU-2 revealed an antibody response which was detected on day 4, increased rapidly, but then decreased after day 10. The maximum mean antibody titers of NYU-2 infected GF and CV mice were 1:80 and 1:50, respectively. The immunoglobulin responses in GF and CV mice infected with 17X were higher than in mice infected with NYU-2.

NIH-white mice that survived the 17X infection were challenged with the NYU-2 strain of P. berghei. All of the mice became infected. No elevations in serum antibody titers or immunoglobulin levels were detected. The 17X strain of P. berghei produced gametocytes whereas the NYU-2 strain did not.

Balb/c mice infected with the 17X strain of P. berghei succumbed to the infection, whereas 50% of C57BL/6 survived. The surviving C57BL/6 were not able to survive a challenge with the NYU-2 strain.

F_1 hybrids from Balb/c and C57BL/6 were inoculated with the NYU-2 strain and none of the mice survived. Other groups of F_1 hybrids were inoculated with 17X; 35-50% of the mice survived. Surviving mice were backcrossed to Balb/c mice and the resulting progeny, F_1 x B, were inoculated with 17X. Of these, 95% survived. F_2 progeny of F_1 survivors infected with 17X also showed a 95% survival. F_1, F_1 x B and F_2 mice, which survived a 17X infection, were challenged with the NYU-2 strain of P. berghei. The survival rate was approximately 85%.

Significance to Bio-Medical Research and the Program of the Institute:

The rapid rise in parasitemia may lead to the synthesis of serum antibody with such low avidity that protection of the host against virulent P. berghei is lacking. The serum antibody produced against the less virulent 17X may be only slightly higher in avidity, protecting the host against homologous strains (17X) but not against heterologous (NYU-2). These results suggest that individuals infected with malaria synthesize antibody of low avidity that is inadequate for protection against virulent forms of the same species of malaria.

The F_1 data suggests either that genetic factors play an important role in resistance to malaria or that some factor transmitted from immunized mother to offspring affects resistance. The data further indicate that a strain of mice can be produced that are resistant to P. berghei which can be used to study the role of humoral and cellular immunity in resistance to malaria.

Proposed Course of Project:

Future studies will focus on the induction of more effective antibody capable of protecting the host against both the 17X and NYU-2 strains of P. berghei.

Honors and Awards: None

Publications:

Hyde, C. L., Master of Science Degree, June 1970. "Immunoglobulin Response and Antibody Synthesis in Germfree and Conventionally-Reared Mice Infected with *Eperythrozoon coccoides*.

Serial No. NIAID - 117-B
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunologic studies in simian malaria

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID, NIH, Bethesda, Md.
Kendall Powers*
P. C. C. Garnham*

Cooperating Units: *Laboratory of Parasite Chemotherapy, NIAID-131

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To study the immune responses of rhesus monkeys (Macaca mulatta) to the simian malaria parasite Plasmodium cynomolgi and of owl monkeys (Aotus trivirgatus) to Plasmodium falciparum. To determine the kinetics of the immune response in blood and sporozoite-induced infections. To determine the effect of various chemotherapeutic agents on the kinetics of the immune response to malaria parasites.

Methods Employed:

Rhesus monkeys were infected with P. cynomolgi by inoculation of sporozoites. Three groups of monkeys were treated with chloroquine for 50, 100 and 150 days, respectively. Another group served as untreated controls. The monkeys were bled twice weekly or weekly depending on the level of parasitemia. Serum antibody titers were assayed by the indirect fluorescent antibody technique. Immunoglobulin levels were determined by a radial-gel diffusion technique. Parasite levels were determined from stained blood smears.

Major Findings:

The parasites were detected in monkeys that were treated with chloroquine. When treatment was stopped, parasitemia appeared 10 days later, and was identical to that seen in the untreated controls. Serum antibody was detected throughout the duration of treatment (mean 1:32). When the monkeys were taken off treatment, the serum antibody titers increased rapidly parallel with the parasitemia. This increase in serum antibody response was also identical to that of the controls. IgM and IgG levels increased slowly throughout the treatment period; but showed a rapid rise after cessation of treatment. When parasitemia and serum antibody titers were increasing with each relapse, IgG levels remained elevated, but the IgM levels rose and fell.

Significance to Bio-Medical Research and the Program of the Institute:

Sterile immunity to malarial infections cannot be induced either in the laboratory or under "natural" conditions. The above results suggest that even when a host's immune system is sensitized to malarial parasites, the antibody synthesized does not protect against a rapid rise in parasitemia. This indicates that the antibody produced is either of low avidity, so that it is relatively ineffective or that the rapid increase in parasitemia may overwhelm the immune system, inducing, perhaps, a partial state of tolerance.

Proposed Course of Project:

Future studies will focus on the induction of more effective serum antibody in *P. cynomolgi* infections in rhesus monkeys. These studies are designed to study the effects of a second antigenic stimulation after resistance has developed in monkeys to *P. cynomolgi*.

Honors and Awards: None

Publications: None

Serial No. NIAID - 117-C
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Separation of different erythrocytic stages of *Plasmodium berghei*

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID, NIH, Bethesda, Md.
William J. Martin*
Allan Rosenthal**

Cooperating Units: *Department of Pathology, Harvard University Medical School, Cambridge, Mass.
**Laboratory of Clinical Investigation, NIAID, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To isolate the various erythrocytic stages of *Plasmodium berghei*. To concentrate and separate the gametocytes from other erythrocytic stages of *P. falciparum*, *P. cynomolgi* and *P. berghei*.

Methods Employed:

Whole blood parasitized with *P. berghei* was obtained from Balb/c mice. Blood was also obtained from owl monkeys (*Aotus trivirgatus*) infected with *P. falciparum* and rhesus (*Macaca mulatta*) monkeys infected with *P. cynomolgi*. The parasites were freed from their erythrocytes and applied to bovine serum albumin (BSA) gradients as well as gradients of a sucrose-epechlorohydrin (SE). Fractions were collected and aliquots stained with Giemsa for microscopic examination.

Major Findings:

Parasites which were freed from their erythrocytes were extremely buoyant and could be separated from white blood cells more efficiently than parasites that were still within erythrocytes. In addition, the gametocytes of *P. cynomolgi* and *F. falciparum*, particularly the latter, could be concentrated and isolated by these procedures. Microscopic examination revealed that the parasites--freed from their erythrocytes--retained normal staining and morphological characteristics. Cell-free *P. berghei* still retained their capability to infect mice.

Significance to Bio-Medical Research and the Program of the Institute:

The separation of malaria parasites, from erythrocytes, which still retain their morphologic characteristics and viability is a significant advance in the preparation of pure malarial antigens. The concentration and separation of gametocytes will allow the use of these stages in determining their role in antigenic stimulation of the host. These procedures permit the isolation of all the blood stages of malaria. Each can be used in the development of new serologic techniques and in the preparation of antigens for immunization of various animal hosts.

Proposed Course of Project:

Studies are in progress which involve the preparation of sufficient quantities of the blood stages of *P. falciparum*, *P. cynomolgi* and *P. berghei*. These preparations will be used for assaying humoral and cellular immunity in malaria.

Honors and Awards: None

Publications: None

Serial No. NIAID - 117-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The immunologic response of animals immunized with trypanosomes

Previous Serial Number: None

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID, NIH, Bethesda, Md.

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	6/12			6/12
Professional:	2/12			2/12
Other:	4/12			4/12

Project Description:

Objectives:

To determine the immunoglobulins produced and classes of antibody synthesized in mice, rats and rabbits immunized with various preparations of *Trypanosoma rhodesiense* and *T. brucei*. To determine the efficacy of the immunization procedures by challenge with live *T. brucei* and *T. rhodesiense*.

Methods Employed:

Animals were infected with *T. rhodisiense* and sera prepared from bleedings taken throughout an infection. Serum antibody was measured by trypanosomal and crithidial agglutination and the indirect fluorescent antibody technique. Trypanosomes were separated from infected blood and washed in saline (0.85% NaCl). The organisms were either heat-inactivated or lysed or frozen-thawed and these preparations were used to immunize mice. Various immunizing dosages were employed and time-intervals between these doses varied. The efficacy of the immunization procedures were determined by challenging with live organisms. The crithidia of *T. rhodesiense* were harvested from *in vitro* cultures and treated in the manner described above.

Sera were obtained from immunized mice and serum antibody to trypanosomes and crithidia determined by agglutination of these forms. The mice were challenged with either live T. rhodesiense trypanosomes or crithidia.

Rabbits were infected with T. rhodesiense to isolate antigenic variants due to the relapsing nature of the infection in these animals. These variants were isolated at 10 day intervals and inoculated into mice.

Major Findings:

Mice immunized with the various extracts were resistant to challenge infections of live T. rhodesiense. All non-immunized controls died in 5 days after inoculation. Two immunizing dosages were required for most of the preparations used. The interval between immunizing doses needed to protect varied according to the amount of material injected.

Mice immunized with crithidia were not resistant to challenge infections of trypanosomes. Serum antibody titers were 1:256 and 1:16 against crithidia and trypanosomes, respectively, using agglutination techniques. Fluorescent antibody procedures showed similar antibody titers to both organisms.

Various strains were isolated from infected rabbits in relapse. One strain demonstrated an altered virulence, i.e. it did not kill mice in 5 days as did the parent parasites and other relapse strains. In addition, this particular strain died more rapidly outside the host than did the other strains.

Significance to Bio-Medical Research and the Program of the Institute:

Active immunization of animals and man against African trypanosomiasis is not practiced because of the lack of suitable immunizing agents. The above results indicate that immunizing agents can be prepared from live trypanosomes of T. rhodesiense and under appropriate conditions, are effective in protecting animals against challenge infections of this organism. The data also indicate that killed whole trypanosomes and certain extracts are efficacious in inducing this resistance. Further studies should define the conditions and preparations which will give the greated degree of protection. The isolation of a strain of T. rhodiense that is not virulent for mice will permit comparison between killed and live attenuated vaccines.

Proposed Course of Project:

Future studies will focus on the isolation and identification of antigen(s) which are most efficacious in inducing protective immunity in animals. The degree of resistance will be determined not only against T. rhodesiense, but against other Trypanosoma species as well. Attention will also be directed to the roles of humoral and cellular immunity in resistance to trypanosomiasis.

Honors and Awards: None

Publications: None

Serial No. NIAID - 118
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synergy in the graft-versus-host response

Previous Serial Number: Same

Principal Investigator: Robert E. Tigelaar

Other Investigators: Richard Asofsky, LMI, NIAID, NIH, Bethesda, Md.
Richard Gershon*
Harvey I. Cantor**

Cooperating Units: *Department of Pathology, Yale University, New Haven, Conn.
**National Institute for Medical Research, London, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	15/12			15/12
Professional:	12/12			12/12
Other:	3/12			3/12

Project Description:

Objectives:

To characterize subpopulations of lymphoid cells which interact in the graft-versus-host (GVH) reaction and to elucidate the nature of this interaction.

Methods Employed:

GVH reactions were produced by inoculation of mouse lymphoid cells from tissues such as spleen, lymph nodes, thymus and peripheral blood (PBL) or combinations of such cells into newborn allogeneic or F_1 recipients. The degree of GVH activity was assayed by two methods. The Simonsen spleen assay, in which the reactivity of the injected lymphoid cells is measured by the degree of spleen enlargement produced in recipient mice was routinely used. A death assay, in which one plots the cummulative mortality of sublethally irradiated recipients of lymphoid cells against time was also employed. Lymphoid cell donors or the cells themselves were treated with a variety of agents with affinity for cell membrane constituents (such as anti-thymocyte serum (ATS), anti-theta, anti-TL, concanavalin, phytohemagglutinin) and the cells then examined for alteration in their behavior in GVH reactions.

Major Findings:

Using the spleen weight assay, it was found that the combination of cells from certain tissues such as thymus and PBL produced GVH reactions that were greater than the sum of the reactivities of these tissues measured separately; i.e. synergy. Both types of cells had to be allogeneic to the recipients in order to obtain increased reactions. Secondary transfer experiments using F_1 strain primary recipients and parental strain secondary recipients indicated that the effector cell or its precursor is represented in excess in thymus cell suspensions while the cell in excess in peripheral blood augments the activity of the former.

Prior treatment of donors with very small doses of ATS did not decrease the GVH activity of any cell population, but severely reduced the capacity of such cells to interact synergistically with normal thymus cells. Treatment of spleen cell donors with larger doses of ATS markedly reduced the GVH activity of such cells; this activity could be substantially restored by adding small numbers of normal PBL.

Lymphoid cells from mice thymectomized at birth were hyporeactive in producing GVH reactions. PBL from such mice produced poor synergy with normal thymus cells; furthermore, spleen cells from such mice were not restored to normal reactivity by addition of small numbers of normal PBL.

Similar results have been obtained using the death assay. The rank order of GVH activity of PBL, spleen, and thymus cells in the death assay was the same as was seen in the spleen weight assay. Combination of numbers of PBL and thymus cells which by themselves produce little or no death consistently yielded very high levels of mortality by 35 days. Spleen cells and PBL characteristically produced death beginning about the end of the second week and continuing for about two weeks; deaths following injection of thymus cells didn't normally begin until late in the third week. Combinations of PBL and thymus cells which gave high levels of cummulative mortality also did not begin to kill until late in the third week. The ensuing disease followed a very rapid course similar to that seen with larger numbers of thymus cells alone; most animals died within a few days, some without overt signs of runting.

The intrinsic specific reactivity of PBL, spleen, and thymus cells from germfree animal donors in GVH reactions was not dramatically lower than that of conventionally-reared mice. Furthermore, PBL from germfree donors interacted with conventional thymus cells as well as did conventional PBL; germfree thymus cells together with conventional PBL reacted as well as did conventional thymus cells.

Incubation of thymocytes from an appropriate strain of mice with complement and an antiserum directed against an antigen (TL) present on the majority of such thymocytes resulted in the recovery of a small residual population of viable cells. All of the direct GVH reactivity of the original thymus cell suspension could be accounted for by the reactivity of this residual population. Furthermore, these residual cells could now interact synergistically with normal thymus cells but not with normal PBL.

Significance to Bio-Medical Research and the Program of the Institute:

Graft-*versus*-host reactions are considered to be a measure of the capacity of lymphoid cell grafts to react against foreign host tissues. The present study has shown that different populations of lymphoid cells can cooperate synergistically in producing a GVH reaction and hence provides insight into a mechanism of cellular immune reactions against foreign tissue. Further delineation of the nature of this interaction is obviously necessary for a clearer understanding of this type of immune response. Study of this model using both conventionally-reared and germfree mice might allow the identification of specific environmental factors necessary for the full development and expression of cellular immune responses. Furthermore, appropriate adjustments of the cell populations involved in cell-mediated immune responses against tumor antigens might enhance the process of tumor rejection of either syngeneic or autochthonous tumors. Similarly, the elimination of small subpopulations of lymphocytes necessary for full expression of normal GVH reactivity may have significance in dealing with the problem of suppression of homograft rejection, and of the graft-*versus*-host reactions which follow bone marrow transplantation.

Proposed Course of Project:

To determine the relationship of various cell membrane antigens such as theta, TL, and Ly present on thymocytes or thymus-derived cells to physiologic properties of those cells. To study the effect on the interacting populations of lymphoid cells in GVH reactions of adult thymectomy of donor mice as well as of adult thymectomy coupled with treatment of donors or cells with ATS, anti-theta, anti-TL, etc. To attempt to identify specific factors responsible for the sensitivity of germfree mice to the suppressive action of ATS. To test the capacity of various synergistic cell mixtures to reject SV-40 induced tumors in mice. To determine whether the cooperation in cell-mediated immune reactions is between cells which are at different stages along a common differeniative pathway or rather is between cells following separate differentiative paths. To determine if the cell responsible for amplification of GVH reactions has physiologic properties similar to the thymus-derived "helper" cell in anti-hapten antibody responses.

Honors and Awards: None

Publications:

Asofsky, R., Cantor, H. I. and Tigelaar, R. E.: Cell interactions in graft-*versus*-host responses. Proceedings of the First International Immunology Congress (Invited Paper).

Serial No. NIAID - 119-A
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Quantitative and qualitative studies on the antibody response to pneumococcal polysaccharides at the cellular level

Previous Serial Number: Same

Principal Investigator: Phillip J. Baker

Other Investigators: Philip W. Stashak and Diana F. Amsbaugh, LMI, NIAID, NIH, Bethesda, Md.
Benjamin Prescott*
Rolf Barth**

Cooperating Units: *Laboratory of Microbiology, NIAID, NIH, Bethesda, Md.
**Department of Oncology and Pathology, University of Kansas Medical Center, Kansas City, Kansas

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	50/12			50/12
Professional:	12/12			12/12
Other:	38/12			38/12

Project Description:

Project Subtitle I: The role of a carrier molecule in the development of an IgG and IgA antibody response to Type III pneumococcal polysaccharide (SSS-III)

Objectives:

To determine whether SSS-III must be bound to a carrier molecule in order to elicit the formation of IgG and IgA antibody.

Methods Employed:

An adaptation of the technique of localized hemolysis-in-gel was used to detect antibody-producing or plaque-forming cells (PFC) specific for SSS-III. Class-specific anti-γ-globulin sera were used to assign the PFC detected to cells making antibody of a given immunoglobulin class. Serum antibody was detected by the specific hemolysis of antigen-coated erythrocytes. Sheep erythrocytes, sensitized with SSS-III by a chromium chloride coupling procedure, were used in all of the above procedures. The types and numbers of SSS-III-specific PFC produced in Balb/c mice immunized with different amounts of either purified SSS-III or of SSS-III bound to various carriers was determined. The latter included: SSS-III bound to a structural component of the bacterial cell wall (killed type-specific pneumococci); SSS-III complexed to methylated bovine serum albumin; or, SSS-III complexed to protamine.

Major Findings:

Although only SSS-III-specific PFC making antibody of the IgM class are produced in mice immunized with purified SSS-III, considerable numbers of cells making $7S\gamma_1$, $7S\gamma_2$ and IgA antibody can be detected in the spleens of mice immunized with SSS-III bound to each of the carrier molecules employed. More IgA-producing PFC were found than both $7S\gamma_1$ and $7S\gamma_2$-producing PFC combined. The maximal number of IgM-producing PFC detected in such mice was no greater than the maximal number obtained in mice immunized with an optimally immunogenic dose of purified SSS-III. Immunization with supra-optimal doses of purified SSS-III regularly results in the detection of fewer IgM-producing PFC (partial immunological tolerance); however, maximal numbers of such PFC continued to be produced in mice given supra-optimal amounts of SSS-III bound to carrier.

Significance to Bio-Medical Research and the Program of the Institute:

Immunological memory is known to be associated with the development of antibody of the IgG class. IgA antibody is the principle type of antibody found in secretions of the respiratory tract. The latter has been shown to play an important role in neutralizing infectious agents at their portal of entry to the body. Neither IgG nor IgA antibodies are produced following immunization with purified SSS-III and prior immunization frequently results in the development of immunological tolerance. Therefore, an understanding of the requisites for the development of these additional classes of immunoglobulins may enable one to devise more effective vaccines to promote long-lasting immunity and to reduce the incidence of pneumococcal pneumonia in certain high-risk populations. The Institute is currently seeking to develop such a vaccine.

Proposed Course of Project:

Efforts are being made to define those conditions which favor the development of IgG and IgA antibody specific for SSS-III. The role played by both types of antibody in confering protection against virulent pneumococci will be assessed. The role of the carrier molecule in the development of immunological memory and tolerance will also be investigated. Other types of carrier molecules will be evaluated for use in the above studies.

Honors and Awards: None

Publications:

Baker, P. J., Stashak, P. W., Amsbaugh, D. F. and Prescott, B.: Characterization of the antibody response to Type III pneumococcal polysaccharide at the cellular level. I. Dose-response studies and the effect of prior immunization on the magnitude of the antibody response. Immunology (in press).

Baker, P. J., Prescott, B., Stashak, P. W., Amsbaugh, D. F. and Barth, R. F.: Immunologic tolerance to pneumococcal polysaccharide as assessed by an immuno-plaque procedure. Ann. N. Y. Acad. Sci. (in press).

Project Subtitle II: Studies on the average avidity of the antibody produced by SSS-III-specific PFC

Objectives:

To determine whether the average avidity of the antibody produced by SSS-III-specific PFC (a) increases sequentially during the course of an immune response, (b) changes as a consequence of prior immunization or with the development of partial immunological tolerance, or (c) is antigen-dose-dependent.

Methods Employed:

The immunological procedures used were identical to those described above. The avidity of the antibody produced by SSS-III-specific PFC was assessed by means of a plaque-inhibition test in which the amount of free antigen required to inhibit the formation of 50% of the specific PFC present was used as an index of avidity.

Major Findings:

Two types of IgM-producing cells can be detected in the spleens of all mice immunized with SSS-III. Although the antibodies produced by such cells differ in their ability to participate in immune hemolysis, no differences in antigen-binding properties were demonstrable. In contrast to the results obtained with most well-studied antigens, no significant changes in the avidity of the antibody produced by SSS-III-specific PFC were evident at various times after immunization and over a 20,000-fold range of antigen dose; neither prior immunization, regardless of the dose of SSS-III employed, nor the induction of partial immunological tolerance, resulted in a change in avidity.

Significance to Bio-Medical Research and the Program of the Institute:

SSS-III is a linear polymer composed of hundreds of identical determinant groups. The participation of a restricted population of lymphoid cells in the antibody response to such an antigen provides one with an unique opportunity to investigate the genetic control of those factors involved in the recognition of antigen and the induction of antibody synthesis by lymphoid cells.

Proposed Course of Project:

Studies will be conducted on the avidity of the antibody produced by SSS-III-specific PFC making antibody of the IgG and IgA class; the results should enable one to determine whether the absence of sequential increases in avidity is characteristic of the class of immunoglobulin (IgM) or the antigen employed. Also, the magnitude of the antibody response to SSS-III is now being evaluated in several different strains of inbred mice to determine which strains can best be used in studies on the genetics control of the antibody response.

Honors and Awards: None

Publications:

Baker, P. J., Prescott, B., Stashak, P. W. and Amsbaugh, D. F.: Characterization of the antibody response to Type III pneumococcal polysaccharide at the cellular level. III. Studies on the average avidity of the antibody produced by specific plaque-forming cells. J. Immunol. (in press).

Project Subtitle III: Factors which regulate the antibody response to SSS-III

Objectives:

To investigate the mechanism whereby antilymphocyte serum (ALS) enhances the antibody response to SSS-III.

Methods Employed:

The immunological procedures used to detect serum antibody and PFC specific for SSS-III are given in preceding sections of this report. Burro anti-mouse lymphocyte serum was administered at the time of immunization with SSS-III; the magnitude of the serum antibody and PFC response obtained was assessed 5 days after immunization. Syngeneic thymus and peripheral white blood cells, obtained from non-immunized mice, were transferred to ALS-treated mice one day after immunization. The rate of antibody synthesis by SSS-III-specific PFC was determined by measuring the rate of appearance of such cells in agarose.

Major Findings:

Treatment with ALS results in a 10-fold increase in the magnitude of the PFC response to SSS-III. In such mice, the serum antibody levels and the rate of antibody synthesis by specific PFC are elevated significantly. However, the infusion of various numbers of syngeneic thymus cells reduces substantially the degree of enhancement attained, while the infusion of peripheral white blood cells further increases the magnitude of the PFC response; with both types of cells effects were proportional to the logarithm of the dose of cells administered.

Significance to Bio-Medical Research and the Program of the Institute:

These studies provide direct evidence for the existance of a homeostatic control mechanism for regulating the antibody response; such a mechanism probably serves to control the rate of antibody synthesis by antibody-forming cells. If such a mechanism can be shown to influence the antibody response to other antigens, it would be of immense value. Of particular concern is the role such cells or their products may play in the prevention of certain undesirable immunological reactions, such as graft rejection and auto-immune diseases, or in determining the level of immunity achieved following immunization.

Proposed Course of Project:

Studies are in progress to obtain more precise information concerning the manner in which both types of regulatory cells exert their effects. An attempt is being made to isolate a product from such cells to determine whether it can likewise suppress or enhance the antibody response to SSS-III and other antigens.

Honors and Awards: None

Publications:

Baker, P. J., Stashak, P. W., Amsbaugh, D. F., Prescott, B. and Barth, R. F.: Evidence for the existance of two functionally distinct types of cells which regulate the antibody response to Type III pneumococcal polysaccharide. J. Immunol., 105: 1581, 1970.

Barth, R. F., Baker, P. J., Stashak, P. W. and Amsbaugh, D. F.: Enhanced antibody production to Type III pneumococcal polysaccharide in mice treated with antilymphocyte serum: Evidence for a cellular regulatory mechanism for the IgM response. Proc. 3rd International Congress Transplant. Soc. (in press).

Project Subtitle IV: The role of cell proliferation in the antibody response of mice to SSS-III

Objectives:

To determine whether cell proliferation and differentiation take place during separate stages of the antibody response to SSS-III.

Methods Employed:

The immunological procedures used for the detection of serum antibody and antibody-forming cells have been described above. Mitotic inhibitors, e.g. hydroxyurea and mitomycin C, were administered at various time intervals following immunization to determine when such treatment was most effective in reducing the magnitude of the PFC response obtained.

Major Findings:

A significant decrease in the magnitude of the PFC response was observed when hydroxyurea or mitomycin C were given during the first two days following immunization with SSS-III; these agents were without effect when administered three to five days after immunization. Previous studies have shown that, during the latter time interval, the rate of antibody synthesis by SSS-III-specific PFC increases progressively. The amounts of hydroxyurea and mitomycin C found to be effective in reducing the magnitude of the PFC response did not alter the rate of antibody synthesis by SSS-III-specific PFC.

Significance to Bio-Medical Research and the Program of the Institute:

Since cell proliferation and differentiation appear to be confined largely to separate stages of the immune response to SSS-III, this permits one to evaluate those factors which influence or regulate each process independently, and, in particular, to determine the extent to which each process is governed by the action of suppressor and amplifier cells.

Proposed Course of Project:

The incorporation of tritiated thymidine by SSS-III-specific PFC will be investigated to provide additional support for the findings obtained thus far. The extent to which other metabolic inhibitors influence cell proliferation and antibody synthesis will also be studied.

Honors and Awards: None

Publications:

Baker, P. J., Stashak, P. W., Amsbaugh, D. F. and Prescott, B.: Characterization of the antibody response to Type III pneumococcal polysaccharide at the cellular level. II. Studies on the relative rate of antibody synthesis and release by antibody-forming cells. Immunology (in press).

LABORATORY OF PARASITIC DISEASES

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Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

Summary statement of research activities July 1, 1970 through June 30, 1971

INTRODUCTION

The consolidation of all research on parasitic diseases from two separate laboratories (Laboratory of Parasite Chemotherapy and Laboratory of Parasitic Diseases) plus a field station in Hawaii (Pacific Research Section) into a single Laboratory of Parasitic Diseases (LPD) has resulted in no obvious administrative or operational problems. However, in spite of continued attrition of permanent personnel the Laboratory is still a large one, it encompasses a wide variety of professional skills and interests, and the geographic separation of two field units from the central facility presents problems or disadvantages. Therefore, much remains to be accomplished in developing a better integrated research program for the Laboratory and taking maximum advantage of the Laboratory's varied resources.

There was no loss of permanent professional personnel during the 1970-71 year. However, the departure of Fogarty Scholar Professor P.C.C. Garnham left a distinct void because his enthusiasm and stimulating presence were felt so keenly. Two guest workers from Argentina, Dr. Gabriel Schmunis, who spent two years with us, and Dr. Ana Szarfman, who worked in LPD for 5 months, both departed in September 1970. Slow but steady losses in secretarial and technical personnel have continued.

Changes in professional personnel were not exclusively subtractions; one of the additions being a medical officer at the Malaria Project in Atlanta, Dr. William Howard. A Visiting Fellow, Dr. Mohamed El-Sayed Omar from Egypt, has joined the Section on Primate Malaria in Chamblee, Georgia, hopefully for three years. Another researcher, Dr. Ruth Pertel, came to us as a Staff Fellow from the National Heart and Lung Institute. Three new Research Associates who joined LPD in July 1970 and remain with us are Drs. Stuart Beeber, Lee Dobbs and Thomas Hyde. Guest worker, Vicki Burdett, a doctoral candidate at Georgetown University, Department of Microbiology, came to work with Dr. Pertel. Dr. Om Prakash from the All India Research Institute of New Delhi, spent 2 months as a guest worker in Dr. Diamond's laboratory.

The peregrinations of Laboratory Staff and visiting colleagues included activities of several staff members abroad on various assignments and a large influx of visiting professionals during the Second International Congress of Parasitology during September 6-9, 1970 in Washington, D.C. Mr. Gam joined Dr. Neva for 3 weeks of intensive work in Panama and Nicaragua on their Chagas' disease project. Other Latin American assignments included Dr. Collins' 2 week visit to El Salvador at the invitation of the Center for Disease Control malaria research group, Dr. Diamond's attendance at an amebiasis symposium in Mexico, Dr. Pacheco's search for an area with human filariasis in Guyana and the Dominican Republic and Dr. Cheever's trip to Brazil as a consultant for Pan American Health Organization. Dr. Cheever also visited Japan for meetings with the U.S.-Japan Program and visited NAMRU-3 in Cairo, Egypt to evaluate possibilities of further studies there. Drs. Pacheco and Beeber went to Nassau in the Bahamas to provide consultation about parasitic infections in children at the Agnes Hardeceker Children's Clinic. Dr. Weinbach attended scientific meetings at the Hugo Theorell Symposium in Sweden for 1 week and the International Congress of Biochemistry in Switzerland for 1 week.

Again during the past year, Dr. Weinbach supervised arrangements for a series of distinguished guest speakers who were invited to the Laboratory for lectures and visits.

SECTION ON PRIMATE MALARIA
CHAMBLEE-ATLANTA, GEORGIA

This Section continues to maintain its concentration on research in malaria. Little recent progress has been made in worldwide malaria eradication efforts; in fact, malaria has recurred in some areas in which it had previously been controlled. Imported malaria in the United States has increased during the past year with most of it being relapsing (vivax) malaria in military personnel returning from Southeast Asia who may or may not have maintained adequate antimalarial chemoprophylaxis. Another continuing problem is that of chloroquine resistance in strains of falciparum malaria in Southeast Asia and South America. No new drug has yet replaced the 4-aminoquinolines for malaria although a few show some promise. The Section continues to place much emphasis on basic studies in malaria since it maintains the philosophy that successful studies on the chemotherapy of this disease are inseparable from basic studies on the infection in the mammalian host and the immune response of the host to the infection.

The consultant arrangement has continued with Dr. Edmund Farrar of the Emory University School of Medicine, and assures a continuing back-up of medical services and consultation for the volunteer program at the Section's Unit on Human Malaria at the penitentiary.

Dr. Mohamed El-Sayed Omar, a Visiting Fellow from Egypt, joined the staff of the Section in November 1970. Dr. Omar did his pre-doctoral training at the Tropen Institute in Hamburg, on the factors which govern the susceptibility of different species of Anopheles mosquitoes to infection with Plasmodium cynomolgi.

Our staff has continued to participate in training programs, in collaborative research efforts and in providing materials for other investigators. Staff members have also been active in providing consultant services and in participation in activities of international and national agencies outside of the National Institutes of Health. More specifically, the Section has maintained and increased its collaboration with the Central America Malaria Research Station in San Salvador in parasitologic, chemotherapeutic, and serologic studies. The Section continues to serve as a reference center for determination of levels and significance of fluorescent antibody titers in suspected malaria cases.

PACIFIC RESEARCH SECTION
HONOLULU, HAWAII

The research program of the Section continues to be oriented geographically and involves the disciplines of virology, bacteriology, protozoology, helminthology, and entomology. Its main objectives remain, 1) to exploit the advantages for epidemiologic research offered by the relatively simple ecologic environment of Pacific Islands, 2) to investigate diseases which are of particular importance on Pacific Island possessions of the United States, and 3) to participate in investigations in Southeast Asia if unique opportunities arise which could contribute to knowledge of diseases which occur in the United States or its territories.

During the year a considerable amount of effort of most staff members was devoted to planning and supervising the construction of the new facilities for the Section at Leahi Hospital. This construction was carried out under the auspices of Contract No. NIH-70-2228 with the Research Corporation of the University of Hawaii at a cost of approximately $450,000 (including the architect's fee and all fixed equipment). The facilities were nearing completion at the end of the fiscal year and it is anticipated that the move to them will be completed before June 30th. The Section will be located immediately adjacent to the present and the planned future laboratories of the Department of Tropical Medicine and Medical Microbiology of the University of Hawaii School of Medicine. It is of interest to note that the Hawaii State Legislature has recently appropriated funds for the first phase of the conversion of this School to a full degree-granting institution.

The Section continues to benefit from having an unusually competent staff - both in professional and supportive categories. The latter personnel (equivalent to 8 full-time positions) are supplied under Contract No. PH43-67-11 with the Queen's Medical Center (at a current annual funding level of approximately $117,000). All persons employed under this contract have agreed to transfer to a similar contract with the Research Corporation of the University of Hawaii when the Section moves to Leahi Hospital.

In addition to performing the research work described in the individual project reports, the professional staff of the Section assisted in teaching medical microbiology and epidemiology to students in the University of Hawaii Schools of Medicine and Public Health.

RESEARCH ACCOMPLISHMENTS

AMEBIASIS

Viruses in amebae: One of the most exciting developments during the past year has been the discovery of evidence for presence of an agent(s) in several strains of axenically cultivated *Entamoeba histolytica*. The agent(s) meet many classical criteria for identification of viruses in that they are filterable, are transmissible in serial passage, possess ultrastructure akin to known viruses, and production of several types of well-defined cytopathic effects. In electron micrographs the agents appear as polyhedral bodies (80mμ) or thin, frond-like filaments within the nucleus and later in the cytoplasm. These findings are still quite preliminary and in the midst of continued study. (Diamond and Mattern of LVD)

Clinical and immunologic studies: A skin test antigen, histolyticin, which is prepared from axenically cultivated E. histolytica, is being evaluated in the United States and in India for its usefulness in the evaluation of amebic disease. Studies in a group of normal individuals suggest that excessive doses may sensitize an uninfected individual and smaller doses must be used for diagnostic or epidemiologic studies. (Kirkpatrick, Lunde and Diamond) A fairly good correlation of positive skin test responses (mainly immediate reactions) with indirect hemagglutination tests was found in patients with various degrees of amebic disease in India. Efforts are being made to study the characteristics of amebae recovered from patients with different clinical varieties of disease. (Om Prakash of India PL-480 Project, Diamond and Lunde)

Ionic requirements for encystment of amebae: Divalent calcium and magnesium have been shown to be required in encystation of Entamoeba. E. invadens which ordinarily does not encyst in axenic culture readily encysts in the presence of lysed Crithidia sp. Crithidia treated with NaEDTA to remove Ca^{++} and Mg^{++} are incapable of inducing cyst formation. On the other hand, Crithidia treated with calcium chelated EDTA induced cyst formation almost equal to that produced with untreated controls. However, other factors are probably also involved in encystment of E. invadens since addition of high concentrations of Ca^{++} and/or Mg^{++} salts had no cyst inducing effect. (Diamond)

TOXOPLASMOSIS

Life cycle and transmission: Toxoplasma gondii is a cosmopolitan parasite of man and numerous animals. In most cases it produces asymptomatic infections but occasionally serious disease occurs. The usual means of transmission in nature are unknown but the finding of an infectious oocyst produced in cats which is infective to man and animals has helped understanding of some epidemiological problems. Further studies to characterize T. gondii have shown that it has developmental stages similar to other coccidian protozoa and has basically the same ultrastructural organization. Attempts to determine the progression of the life cycle of the parasite in the cat are in progress. Inoculation of oocysts vs inoculation with cysts of T. gondii orally intc cats produces somewhat different infections. Oocyst output is much higher in cyst-induced infections and, in many cases, oocyst-induced infections do not produce any oocysts. Antitoxoplasma antibody titers tend to be much higher after cyst inoculation and cats are resistant to challenge whereas reinfection can occur after oocyst inoculation. This may have epidemiological importance since cats may be commonly infected by eating mice which harbor T. gondii cysts. (Sheffield and Melton)

Immunological studies: Production of host resistance to virus infection by a toxoplasma-related organism, Besnoitia jellisoni and the converse have been shown but the mechanism was unknown. Recent studies with B. jellisoni and Semliki Forest virus demonstrate that mice infected with the former are resistant to a normally lethal challenge of the latter. This resistance is thought to be due to activated macrophages. (Lunde)

Biochemical taxonomy of toxoplasma and chemotherapy of toxoplasmosis: Studies of the DNA of representative species of coccidia are in progress in order to determine the genetic relationship of T. gondii to other coccidia. These organisms have a smaller genetic potential and less DNA complexity than most higher organisms. T. gondii differs from members of the genus Eimeria in that it has less DNA per cell and higher percentage of guanine and cytosine in its DNA. These are the first studies of the complexity of DNA of obligate intracellular protozoal parasites. (Gelderman) Chlorinated analogues of lincomycin, U-24 and U-21, have been shown to exert chemotherapeutic activity against toxoplasma infection in mice. (Lunde, Powers, McMaster and Finerty of LMI)

TRYPANOSOMIASIS

Survey for infection and disease in Nicaragua: An epidemiologic survey for prevalence of T. cruzi infection and Chagas' disease was carried out in selected areas of Nicaragua. The presence of two vector bugs, mainly R. prolixus and T. dimidiata was found, with Trypanosoma rangeli as well as Trypanosoma cruzi being present in the former. Complement-fixation tests on 550 sera showed a high prevalence of infection with T. cruzi, but the results of clinical evaluation for presence of Chagas' disease are not yet available because the study is still in progress. (Neva)

Tissue culture antigen from T. cruzi: The antigen prepared from tissue cultures infected with T. cruzi, and containing predominantly amastigote and trypomastigote forms, was used in comparative CF tests with other antigens. The results with the tissue culture antigen were qualitatively very similar to those obtained with a well-characterized protein antigen, but titers with the tissue culture antigen were generally higher. A variable degree of cross-reactions between the tissue culture CF antigen and sera from a few patients with leishmaniasis was found, but this matter needs further study. (Gam and Neva)

Analysis of intracellular infection with T. cruzi: A detailed analysis of the sequence of events involved in infection of cells by T. cruzi is under study. This is carried out by utilizing a specially designed culture chamber that permits direct observation by phase microscopy, photography and various optical measurements of cellular and subcellular phenomena. Five different phases of the intracellular cycle of the parasite have been defined (penetration, "reorganization", reproduction, differentiation, and escape). In such a system epimastogotes or trypomastigotes of T. cruzi invaded mouse peritoneal macrophages readily but were destroyed by the cell; the fate of the infected cell was dependent upon the growth phase of infecting epimastigotes. (Dvorak and Schmunis) The infection of beef-embryo cells by T. cruzi is being studied quantitatively with variable sized inoculum to define the precise nature of the host-parasite interaction. It appears that the results can be depicted in a mathematical model. (Hyde and Dvorak)

Interferon in T. congolense infections: The interferon inducers, Poly I . Poly C and, in some cases, Poly A . Poly U protect mice infected with selected variants of T. congolense. The fact that protection could be reversed by administration of the immunosuppressant, cyclophosphamide, indicates that the protection of interferon inducers was mediated via a mechanism of enhancing immunologic responsiveness rather than interferon production. (Herman)

Calcium in trypanosome lesions: Calcium accumulates in the livers of mice infected with *T. cruzi* but the amounts detected do not appear to influence oxidative activity when tested in a cytochrome oxidase system. (Mercado)

MALARIA -HUMAN

Human malaria in the owl monkey: The owl monkey, *Aotus trivirgatus*, has been infected with three additional strains of *Plasmodium vivax* (Salvador II, South Vietnam III, and NICA) bringing the total number of strains of vivax malaria up to nine which we have found to infect this simian host. Two new isolates of *Plasmodium falciparum* from Panama and South Vietnam were established in owl monkeys but could not be serially passaged. *Plasmodium ovale* continues to be the only species of human malaria with which we can not infect this experimental host. The human malaria parasites in the owl monkey are infectious to mosquitoes, and man can be infected through the bites of these mosquitoes. The Panama strain of *P. vivax* was transmitted from man to an owl monkey by sporozoite inoculation suggesting that on rare occasions this monkey might serve as a reservoir of infection under optimal conditions. Also of major significance was the finding of exoerythrocytic liver stages of human vivax malaria in the owl monkey. (Contacos and Collins)

Characterization of human strains: In collaboration with the Central America Malaria Research Station in Salvador, four strains of Central American vivax malaria have been characterized for their relapse patterns and drug sensitivity. All four strains were found to have latent periods between primary attack and first relapse activity - the first relapse occurring approximately at 150 days after exposure to infection or later. All strains of vivax malaria were found to be sensitive to standard antimalarial drugs. (Contacos, Krotoski, Howard and Collins)

Relationship of parasite schizogony to fever: As a start to reinvestigating the old question of what causes fever in malaria, a more precise determination is being made of the time-course of parasite schizogony in relation to onset of malarial paroxysm. Several patients have already been studied at 15 minute intervals in an effort to determine a time point or interval during which maximum rupture of schizonts occurs. White cell, platelet counts and serum complement levels are also being followed at less frequent intervals. Methods for the study are still being standardized but preliminary results indicate that it should be possible to establish the time interval between schizont rupture and ensuing febrile response in well synchronized non-falciparum infections with reasonable precision. (Neva, Krotoski and Howard)

The problem of hepatitis: in some volunteers whose infection was initiated by inoculation of blood resulted in great efforts to elucidate the cause. In spite of tests for Au antigen and/or liver function in prospective donors and recipients the exact cause of inoculation hepatitis remains obscure. (Krotoski, Howard and Neva)

MALARIA CHEMOTHERAPY

Lincomycin analogues: Studies with chlorinated lincomycin analogues were extended to P. knowlesi in rhesus monkeys and to a highly chloroquine-resistant human strain of P. falciparum in owl monkeys. In these models the results with the U-24 analogue are encouraging and further tests to define the effective dosage range are in progress. (Powers and Jacobs) The U-21 analogue, Clindamycin, which is already commercially available for treatment of bacterial infections, has had limited tests in man against P. vivax and P. ovale with less satisfactory results because of its slow action against blood form parasites. (Contacos, Krotoski and Howard)

Other research on antimalarials: Since chloroquine is frequently used in experimental work on malaria in monkeys the relationship of plasma levels to curative activity vs P. cynomolgi was studied. In blood-induced infections single doses of 10 mgm/kg chloroquine administered I.M. were not uniformly curative, but doses of 20 mgm/kg cured all infections. (Jacobs) Studies with drug-resistant strains of rodent malarias (P. berghei and P. vinckei) showed no antigenic differences as detected by parasitemia after direct challenge between a chloroquine-resistant vs drug-sensitive strain of P. berghei, and chlorinated lincomycin compounds were found effective vs multi-drug-resistant P. berghei. (Jacobs)

MALARIA-SIMIAN

Exoerythrocytic tissue stages and relapse: Studies have continued on the exoerythrocytic (EE) or tissue stages of simian malaria parasites. Exoerythrocytic stages of Plasmodium simium, a tertian parasite of South American monkeys, were observed for the first time from two experimental simian hosts (squirrel and owl monkeys). The EE stages of P. knowlesi were intensively studied to determine their rate of growth. These studies on tissue stages of malaria provide a number of host-parasite models for study of compounds suspected of having causal prophylactic and/or radical curative activity against malaria. (Collins and Contacos)

Studies on the phenomenon of relapse indicated that sporozoite-induced infections of Plasmodium semiovale will relapse from two to six months period of time. This points up a biologic relationship between this species and Plasmodium fieldi in monkeys and P. ovale in man; just as P. cynomolgi in monkeys relates to P. vivax of man. (Collins and Contacos) The long-term relapse study with 3 strains of P. cynomolgi was completed and has shown that the number and longevity of relapses is a function of numbers of sporozoites injected. This would support a concept of latency of some EE stages to explain delayed patency and late relapses, instead of the original proposal in 1948 by Shortt and Garnham of continued cyclic development of EE stages in the liver. (Warren, Powers, Garnham)

A monograph on "The Biology of the Primate Malarias" has been completed and is in press. (Contacos, Collins, Warren and Coatney)

IMMUNOLOGICAL STUDIES OF MALARIA

Immunization vs malaria: Analysis of antigens of simian malaria parasites by immunoelectrophoretic means is continuing as are efforts to immunize simian hosts with various types of antigen. Little or no success has been encountered in these immunization studies; the patterns of parasitemia in "immunized" and control animals are much the same. To date, no complete protection against malaria infection has been observed after immunization using extracts, parasitized erythrocytes, free asexual parasites, serum soluable antigens, or formalinized sporozoites. (Contacos, Krotoski and Collins)

Immunization of monkeys using X-irradiated sporozoites of *Plasmodium cynomolgi* also failed to completely protect monkeys from infection but did produce a delayed prepatent period possibly indicating a reduction in the number of sporozoites invading liver tissue at the time of challenge. In one animal, the resultant parasitemia was also reduced over that of the control monkey. (Contacos and Collins)

Epidemiological studies using the IFA technique: Studies in collaboration with the Central America Malaria Research Station in El Salvador on inhabitants of the Mato Grosso area of Brazil indicated that the IFA technique gave a more accurate picture of the malarial experience of population than that obtained by a malaria survey. (Collins)

Role of specific antibody in resistance to malaria: Studies on the effect of horse antihuman thymocyte globulin (HAHTG) on infections of *Plasmodium fieldi* in macca mulatta monkeys revealed that treated animals had much higher parasitemias than did control animals. Monkeys inoculated with HAHTG previously absorbed with monkey red blood cells exhibited parasitemias much lower than did control animals. (Collins)

From experiments in which sporozoite-induced *P. cynomolgi* infections in rhesus monkeys were suppressed with chloroquine it appeared that fluorescent antibody was not inhibited. Even though peripheral parasitemia was absent the drug-suppressed animals developed antibody at the same time as controls, although antibody levels soon rose to higher levels in unsuppressed controls. Serum antibodies produced prior to patency had little or no effect upon the subsequent course of infection after drug suppression was stopped. (Powers and Finerty)

Interaction of malaria with other infections: Chikungunya and influenza virus, but not NDV, protect chick embryos against the exoerythrocytic form of *Plasmodium gallinaceum*. The possible roles of both low levels of interferon and viral interference may be considered in this protection. (Herman) Passaging of blood from rats exhibiting the *P. berghei* paralyzing syndrome in white mice appears to restore the optimal conditions for cultivation of M. neurolyticum, the organism suspected to be the cause of the paralysis. Isolation from germfree mice must be obtained, however, before this organism can be ultimately linked with this synergism. (Mercado)

ENTOMOLOGICAL STUDIES

Transmission through mosquitoes: was demonstrated for eleven strains or species of primate malaria, with a full study of details of prepatency and infection in the simian hosts. Newer isolates of P. vivax and P. falciparum continue to infect various species of mosquitoes. Long term mosquito feeding studies indicate: 1) that infections of human malaria in the Aotus monkey will infect mosquitoes almost continuously for at least 8 months and possibly longer, and 2) that infection can be transmitted by these mosquitoes. (Collins)

FILARIASIS

Host-parasite relationships: Much of the disease associated with filarial infection is believed to be an alteration of host-parasite relationships. Modification of these relationships was attempted by exposing hosts to microfilariae in utero or by treatment with anti-thymocyte serum. Duration of microfilaremia was not prolonged by either treatment suggesting that the primary clearance of microfilariae is not an immunological response.

Serologic studies: Diagnosis of filarial infections when microfilaremia is absent is presently based almost totally on clinical evidence. Lack of specificity of serodiagnostic methods currently in use makes these of little help. Metabolic products of living infective-stage larvae used to coat tanned erythrocytes appear to be antigenically specific with sera from superinfected animals, but do not react with sera from animals given a single injection of larvae. This serologic system is being studied with various species of filaria and various stages in the life cycle. (Pacheco)

SCHISTOSOMIASIS

Liver fibrosis and glomerulitis in experimental schistosomiasis: Chimpanzees heavily infected with Schistosoma japonicum regularly developed liver fibrosis indistinguishable from that in persons with schistosomiasis. Several factors affecting the development of this serious complication were identified. Several chimpanzees with hepatic fibrosis also developed glomerulitis, a complication recently described in persons with severe schistosomiasis. (Sadun, von Lichtenberg, Erickson, Cheever)

Carcinoma of the urinary bladder: was seen in two monkeys infected with S. haematobium. Hydroureter and hydronephrosis were produced in several species of monkeys and baboons infected with this schistosome: the chimpanzee was the only previously known animal model for studying these complications of urinary schistosomiasis, which are important in infected persons. (Kuntz, Myers, Cheever)

The susceptibility of snails to schistosome infection: was shown to be genetically determined, but susceptibility was not linked with visible genetic markers. Stocks of snails resistant to S. mansoni infection were developed but these snails still carry susceptible genes which should be eliminated before attempts are made to replace a susceptible population in nature with resistant snails of the same species. (Richards)

Studies of protozoa and bacteria infecting molluscs: were continued to better understand molluscan diseases which affect experiments and diseases which might be useful for biological control. A new species of microsporidian infecting the snail vector of S. mansoni was described. (Richards)

BIOCHEMICAL AND BIOPHYSICAL STUDY OF TAPEWORMS

Functional role of non-heme iron in cestode respiration demonstrated: Non-heme iron is an integral part of the respiratory chain of mammalian mitochondria. Chemical analysis of mitochondria isolated from three species of cestodes revealed that the ration of non-heme to heme iron exceeds that found in mammalian mitochrondria. Spin resonance studies demonstrated that the characteristic non-heme iron signal (g=1.94) developed when the cestode mitochondria were respiring on a-glycerophosphate. In the presence of iron-chelators both the mitochondrial respiration and the spin resonance signal were abolished, providing for the first time conclusive evidence the functional non-heme iron proteins are present in parasites. (Weinbach and Dvorak)

BIOCHEMICAL STUDIES OF HUMAN MITOCHONDRIA

Mitochondrial defects found in biochemical studies of human tissues: Considerable experience and biochemical information has been gained during the past decade from studies of mitochondria isolated from various animal tissues. Application of this experience to several collaborative projects related to clinical studies of myopathy (King, Kark, NINDS); acute, intermittent porphria, (Tschudy, NCI); platelet metabolism (Wolfe, NIAMD) have demonstrated that biochemical abberations of mitochondrial function are associated with human disease. (Weinbach)

GROWTH AND DIFFERENTIATION OF PARASITIC AND FREE-LIVING NEMATODES

The transitional point of the life cycle of parasitic nematodes: is being investigated utilizing the techniques of genetics, biochemistry, and neurobiology. Clonal lines of axenic, free-living nematodes with mutational changes in their development that mimic the life cycle of parasitic nematodes are being studied to determine factors affecting development. (Pertel)

Neurobiology: The mode of action of the DuPont nematodide 1410 was established as a potent inhibitor of the nematode acetylcholinesterase. (Pertel)

Nucleic Acids: A number of satellites of DNA have been established for nematodes, both free-living and parasitic. Satellite DNA's of young and reproducing nematodes are very different. (Burdett, Dobbs and Pertel)

COMPETITION BETWEEN AEDES ALBOPICTUS AND AEDES POLYNESIENSIS

One year after the release of Albopictus on Taiaro atoll, both adult and larval specimens of this species were collected in an area several miles wide. Although this indicates that the species probably has become established on the island, in numbers of individuals, it still comprises a relatively small part of the total mosquito population. In retrospect, it appears that considerable time elapses between each mosquito generation on the island because of the sporadic rainfall of short duration and the high evaporation rate. Thus, in view of the relatively small numbers of Albopictus originally released, it appears that further observations over an unknown period of time will be needed to determine the ultimate outcome of the experiment. Because of these findings, it is planned to proceed with a second experimental release on an accessible volcanic island with a more appropriate climate. The accessibility of the island should permit the release of many more Albopictus over a much longer period of time than did the original experiment and hence it should be apparent relatively rapidly whether or not this species will in fact displace Polynesiensis in nature as it does in the laboratory. Observations also will be continued on Taiaro. (Rosen)

EOSINOPHILIC MENINGITIS

No new research was carried out during the year but considerable effort was devoted to preparing data already accumulated for publication. (Rosen)

TOXOPLASMOSIS

This project is still focused on determining the role which domestic cats play in the maintenance of the infection in nature and its transmission to man. Significant findings during the year included, 1) the demonstration of oocysts in the feces of naturally infected cats, 2) the difficulty of infecting cats with oocysts, with the implication that intermediate hosts play an essential role in the maintenance of the infection in nature, and 3) the absence of infection in other animals in areas where cats are absent. (Wallace)

SEASONAL GASTROENTERITIS IN THE PHILIPPINES

The nearly completed investigations on this project failed to yield an explanation for the illnesses despite careful study, the use of the latest laboratory techniques, and the feeding of fecal material from patients to 22 human volunteers. It was shown that approximately 20% of persons with acute symptoms of gastroenteritis during the annual outbreaks went on to develop signs, symptoms, and laboratory findings of mild tropical sprue. Although the findings with respect to etiology were negative, the data derived from this study should provide valuable background information on both the acute illness and its chronic sequelae in the event that promising hypotheses or new laboratory techniques become available in the future. (Dean)

ENTEROTOXIN PROCUCING *E. COLI*

In view of recent interest in the role of enterotoxin-producing strains of *E. coli* as possible causes of diarrheal disease in both adults and children, a study designed to evaluate the role of such organisms in pediatric diarrheal disease in Hawaii and elsewhere was undertaken. As part of this investigation, an attempt was made to improve the current cumbersome biologic test for such toxins, which now involves the use of ileal loops of rabbits. Encouraging results have been obtained with a test employing infant mice. Should this test prove satisfactory, a significant savings in time and expense will be achieved. (Dean)

ARBOVIRUSES

The work on this project was devoted entirely to dengue viruses which currently are important causes of human disease in Asia, the Caribbean area, and Africa. Major findings during the year were, 1) the demonstration that the intrathoracic inoculation of susceptible species of mosquitoes was more sensitive by several orders of magnitude than other laboratory methods for the detection of unmodified strains of dengue virus, 2) that a single infected mosquito can yield more than 100×10^6 infectious doses of virus, 3) that mosquitoes are far less susceptible to dengue viruses by ingestion than by intrathoracic inoculation, and 4) that various species of the subgenus stegomyia are equally susceptible to infection by intrathoracic inoculation and far more susceptible by this route of inoculation than are non-vector species of *Culex* and *Armigeres*. The intrathoracic injection technique holds considerable promise for use in studies of the pathogenesis of dengue viruses in primates, and particularly in studies of the pathogenesis of dengue hemorrhagic fever. Until now, all types of laboratory studies of dengue have been handicapped greatly by the absence of sensitive animal hosts (other than primates) or cell culture systems for the assay of dengue viruses unmodified by adaptation to mice or cell cultures. The intrathoracic injection technique also offers a method of producing large amounts of unmodified virus for vaccine studies. (Rosen)

Serial No. NIAID 120
1. Parasitic Diseases
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Administration, Research Planning and Coordination from the Office of the Chief

Previous Serial No.: Same

Principal Investigator: Franklin A. Neva

Other Investigators: Allen W. Cheever

Cooperating Units: Section on Clinical Parasitology, Laboratory of Clinical Investigation, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	125/12			125/12
Professional	8/12			8/12
Other	117/12			117/12

Project Description:

This project furnishes administrative and supervisory services to research projects in the laboratory as follows:

Research plans are discussed with various investigators and overall research activities are coordinated. Travel of personnel is arranged and secretarial services are provided. Assistance is given in the preparation, editing, and reviewing of manuscripts and reports. Requisitioning and supervision of equipment and supplies and maintenance of the library is provided. Consultation and the liaison activities are maintained with individuals, academic organizations, and other branches of the Federal Government in matters pertaining to the activities of the laboratory. Arrangements are made for supervision of research workers who are in a training status. Centralized facilities and supervision are provided for care of experimental animals used by investigators.

Liaison is maintained with the Section on Clinical Parasitology in the Laboratory of Clinical Investigation with reference to diagnostic services or investigative work that is carried out with patients at the Clinical Center.

The administrative adjustments relative to incorporation of the former Laboratory of Parasite Chemotherapy and the Pacific Research Section into this Laboratory have been made and operational channels appear to be functioning adequately. Both field laboratories continue to operate with minimal administrative restrictions from Bethesda.

Laboratory and office space is being renovated at a new location for the Pacific Research Section. The move from Queen's Hospital to the renovated facilities at Leahi Hospital will probably occur around July 1971. The new location for the Pacific Research Section is in the same building as the Department of Microbiology of the University of Hawaii School of Medicine.

A close working relationship between certain members of the Laboratory of Parasitic Diseases and the Laboratory of Clinical Investigation continues. A Section on Clinical Parasitology with Dr. F. A. Neva as Head has officially been established within the Laboratory of Clinical Investigation. Dr. Peter Franck came from the Middle America Research Unit in Panama to work in the new Clinical Parasitology Section of LCI. Dr. Franck attends many seminars and conferences at LPD, and facilities of LPD are available for Dr. Franck's research work. In addition, one of the Clinical Associates from LCI, Dr. Theodore Nash, has elected to pursue a research project on schistosomiasis in collaboration with Drs. Cheever and Neva.

Animal care and technical assistance with animal experiments are provided by 5 technicians under the supervision of Mr. Paul Koger.

Honors and Awards:

Franklin A. Neva

A member of Editorial Board of the American Journal of Tropical Medicine and Hygiene; resignation submitted and accepted in December 1970.

Member of the Medical Advisory Board of the Leonard Wood Memorial.

Member of Board of Directors of the Gorgas Memorial Institute of Tropical and Preventive Medicine, Inc.

Serial No. NIAID 121-C
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The biology and control of the snail intermediate hosts of schistosomes

Previous Serial Number: Same

Principal Investigator: Charles S. Richards

Cooperating Units: None

Man Years

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To study molluscan genetics with particular attention to applications to control measures. To study prospective agents for biological control of intermediate hosts of the schistosomes. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the contributions and complications of molluscan diseases and molluscan genetics in biomedical research. To study the causes of abnormal tissue growths in mollusks.

Methods Employed: Selection and crossing experiments are conducted to find additional genetic markers, to characterize linkage groups, to increase knowledge of mechanisms of molluscan genetics, and to investigate further the genetic aspects of molluscan susceptibility to infection and to molluscicides.

Parasites of mollusks are studied to determine: their identity, molluscan host range, mode of transmission, localization and pathology of infection, and interrelations with schistosome infections.

Genetic studies, fresh dissections, and stained serial sections are employed to determine the causes and pathology of abnormal tissue growths in mollusks.

Major Findings: Studies on the genetics of susceptibility of *B. glabrata* for infection with *S. mansoni* were continued, including genetic factors modifying the simple adult susceptibility factor. In some clonal stocks early development of the parasites in the hepatopancreas was frequently observed. Migrating daughter sporocysts were observed as early as the ninth day after infection, and in some cases miracidia penetrated to the hepatopancreas where primary sporocysts then developed. In other clonal stocks delayed development of the parasite was observed. Developing primary sporocysts were first observed in feet and tentacles several months after exposure to miracidia. This apparent prolonged dormancy of the parasites in active snail hosts appears similar to the reported dormancy of juvenile parasite stages in estivating snail hosts.

Susceptible and refractory genetic stocks of *B. glabrata* are being used in collaborative studies with Dr. Gelderman and Mr. Keister in an attempt to demonstrate biochemical differences between the stocks, to eventually explain the basis mechanisms of susceptibility.

Crosses are in progress between *B. glabrata* from St. Lucia (juvenile and adult susceptible to both St. Lucian and Puerto Rican *S. mansoni*) and two of our genetic lab stocks of *B. glabrata* (both refractory to St. Lucian *S. mansoni*; a blackeye stock juvenile susceptible but adult refractory to Puerto Rican *S. mansoni*, and an albino stock juvenile and adult susceptible to Puerto Rican *S. mansoni*). Preliminary results indicate the hybrid F1s are juvenile refractory to the St. Lucia parasite.

The search for visible mutant characters to serve as genetic markers was continued. A form of abnormal tentacle growth termed "antler tentacle", was demonstrated to be regulated by a single recessive gene, showing simple Mendelian inheritance. This tentacle abnormality usually appears after maturity and develops progressively with age, is independent of environmental conditions, and appears to be irreversible. Susceptibility to infection with *S. mansoni* does not appear to be linked with any of the genetic markers so far identified.

Occasional individuals with an everted preputium have been observed in several snail stocks. Selection has resulted in stocks breeding true for this character. In some cases the everted preputium is branched or swollen so that it is no longer retractable. In one clonal stock the everted preputium has a protruding tissue plug, and is ineffective in cross-fertilization. Inbred, this stock can reproduce only by self-fertilization in individual mated with a normal stock snail serves as a female only, cross-fertilization being one-sided.

Polyps and extra tissue folds on the proximal side of the pseudobranch have been observed in several stocks of *B. glabrata*. Frequencies suggest inheritance of these abnormal growth characters.

Studies were continued on the microsporidian infecting *B. glabrata* which has been described as a new species of *Coccospora* in collaboration with Dr. Sheffield. Showing a high prevalence as a relatively harmless infection of the wall of the intestine, this protozoan causes more extensive infection and severe pathology in some *B. glabrata*, including formation of laminated mats on the inner surface of the shell. Selection and crossing indicates the host-parasite relation resulting in severe pathology is genetic.

Studies on amebae associated with mollusks were continued. Snails are occasionally observed with an impacted intestine. A few *B. glabrata* so affected have been dissected, revealing a compact mass of fecal material surrounded by a dense growth of filamentous fungus harboring numerous *Naogloria* amebae.

Flagellates of the genus *Cryptobia* from the Taiwan planorbid snails Segmentina hemisphaerula and *Gyraulus spirillum* are being studied in collaboration with Dr. Dvorak, particularly with regard to a phenomenon involving repeated reversal of the body orientation of the protozoa.

An intracellular bacterial parasite of *Bulinus jousseaumi*, an intermediate host of *Schistosoma haematobium*, is being studied in collaboration with Dr. Roger M. Cole (LM). Electron microscopy reveals that this non-motile gram-negative red has a circle of straight, hairlike appendages at one end, larger than and structurally different from flagella.

Significance to Biomedical Research and the Program of the Institute: *B. glabrata* is an excellent model for studies in molluscan genetics. Information already obtained indicates the importance of genetics as a variable in experimental research. Change in susceptibility to infection and pearl formation, genetic factors associated with maturation, are examples of physiological changes occurring at this state of development. Ranges in size and age at onset of egg laying, influence by inheritance, emphasize the need for careful selection of test snails in research; and awareness in analyzing results.

Need for evaluation of the role of genetics in snail control continues. Refractory *B. glabrata* may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Present knowledge of the genetics of susceptibility of juvenile and adult B. glabrata to infection with *S. mansoni* indicates the need for additional information before field trials are attempted.

Development of clonal stocks of various genetic types of susceptibility provides material for cytogenetic studies, and biochemical studies to determine the basic mechanisms affecting the host-parasite relationships.

Microsporidia, amebae, and bacteria cause extensive damage in some snail hosts. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites in order to plan, conduct, and interpret experimental research on snails with validity.

Information on a variety of abnormal tissue growths observed in *B. glabrata*, many of which are genetic, should help in analyses of tumors of mollusks collected in the field.

Proposed Course of the Project: Genetic studies on *B. glabrata* will be be continued in search of additional genetic markers, added by chemical induction of mutations; studies on genetics of susceptibility of *B. glabrata* for *S. mansoni* infection will be continued, particularly delayed infections and factors involving additional *B. glabrata* stocks and *S. mansoni* strains. In collaboration with Dr. Gelderman and Mr. Keister studies on the biochemical differences between susceptible and in susceptible snails will be continued.

Studies of parasites infecting mollusks will be continued; particularly microsporidia, amebae, and bacteria; including relations to the molluscan hosts, affects on other parasites, potential infection of man, and as a varial factor in experimental research.

Abnormal tissue growths will be studied by fresh dissections and culturing, serial sections, genetic selection and crossing, and environmental manipulation to determine causal factors.

Honors and Awards:

Participation in Colloqium on Biology and Disease Potential of Small Free-living Amoebae, 2nd International Congress of Parasitology, Sept. 6-12, 1970, Washington, D. C.

Participation in workshop on Intramolluscan Development of larval Schistosomes: Physiology, Pathology, and Host specificity, U.S. Parasitic Diseases Panel, U.S.-Japan Cooperative Medical Science Program, Harvard School of Public Health, April 26-27, 1971.

Publications:

Richards, C. S.: Genetic studies on *Biomphalaria glabrata*: tentacle and eye variations. *Malacologia*, 9: 327-338, 1969.

Richards, C. S.: Genetic studies on *Biomphalaria glabrata*: mantle pigmentation. *Malacologia*, 9: 339-348, 1969.

Richards, C. S.: Pearl formation by *Biomphalaria glabrata*. *J. Invertebrate Pathology*, 15: 459-460, 1970.

Richards, C. S.: Genetics of a molluscan vector of schistosomiasis. *Nature*, 227: 806-810, 1970.

Richards, C. S.: *Biomphalaria glabrata* genetics: spire formation as a sublethal character. *J. Invertebrate Pathology*, 17: 53-58, 1971

Richards, C. S.: *Urceolaria viridis* n.sp., a ciliate (Peritridia, Mobilina) with elongate symbiotic green algae. *J. Protozoology* (In press).

Serial No. NIAID 121-H
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The pathogenesis of schistosome infections in mammalian hosts.

Previous Serial Number: Same

Principal Investigator: Dr. Allen W. Cheever

Other Investigators: Dr. Kendall G. Powers

Cooperating Units: Laboratory of Microbial Immunity, NIAID (Dr. Finerty) Laboratory of Clinical Investigation (Dr. Nash) Department of Medical Zoology, Walter Reed Army Institute of Research (Drs. Sadun and Erickson); Department of Pathology, Peter Bent Brigham Hospital, Boston, Mass. (Dr. von Lichtenberg); Department of Parasitology, Southwest Foundation, San Antonio, Texas (Drs. Kuntz, Myers); East African Institute for Medical Research (Miss Obuyu, Dr. Eyakuze).

Man Years

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	36/12			36/12
Professional	12/12			12/12
Other	24/12			24/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PH 43-68-1337	A. Cheever	Bionetics Research Laboratories	Dr. S. Sibinovic	$23,000

Serial No. NIAID 121-H

Project Description:

Objectives: The interactions between various mammalian hosts and schistosomes of medical importance are studied, with particular reference to the dynamics of egg production by the parasite and the excretion or destruction of eggs by the host. The pathologic lesions in various hosts are studied in relation to the above variables and to physiologic changes, such as the development of portal hypertension and collateral circulation. The influence of host immunity and hypersensitivity on tissue reactions and the ability of the host to dispose of eggs are studied. The effects of the host reaction on the parasite, *in vivo* and *in vitro*, are also examined.

Methods Employed: Laboratory rodents and primates infected with *S. mansoni*, *S. haematobium* and *S. japonicum* are studied during the course of infection and are sacrificed at various intervals after infection. Observations made during the course of infection are correlated with parasitological and pathological findings at the time of autopsy.

Major Findings: *S. haematobium* infection in nonhuman primates: Low grade transitional cell carcinomas have been found in one talapoin and one capuchin monkey infected with S. haematobium for about one year and transitional cell lesions judged to be precancerous were seen in two squirrel monkeys infected for 8 months. No spontaneous bladder cancers have ever been reported in monkeys.

Hydroureter and hydronephrosis have been observed repeatedly in three species of baboons and three species of monkeys infected with *S. haematobium* (Kuntz, Myers, Cheever). Similar studies have been carried out in Tanzania (Obuyu, Cheever).

S. japonicum infections in chimpanzees: Symmers' clay pipestem fibrosis of the liver has been produced consistently in chimpanzees 3 months after exposure to *S. japonicum*. Half of the animals currently being studied will be treated to determine the reversibility of the pipestem fibrosis. In this early stage, the chimpanzees develop moderate portal hypertension which is functionally and anatomically presinusoidal (intrahepatic pressure is normal). Hepatic blood flow was only slightly reduced in animals with marked portal obstruction. The chimpanzees with pipestem fibrosis also develop glomerulitis.

S. japonicum infections in monkeys: Infections in several species of monkeys were initiated in an attempt to find a more freely available model for the study of pipestem fibrosis, and also because of the marked differences in the reactivity of monkeys and chimpanzees to *S. mansoni*. One species of monkey shows early lesions similar to those seen in the chimpanzee, but no clearcut pipestem fibrosis was seen in monkeys killed three months after infection.

S. mansoni infections in African green and rhesus monkeys: Monkeys in this long term study have now been folled for 30 months. The number of eggs passed in the feces dropped about 50% during the first year after infection but has no changed since that time. Monkeys repeated reexposed to infection developed marked but not complete resistance to reinfection. Monkeys challenged more than 2 years after their first exposure were much less resistant to reinfection (Cheever).

Circulating antigen in S. mansoni infected animals. Exposure of rhesus monkeys to high and low numbers of S. mansoni cercariae has shown a virtual absence on immune response (worm death) to an initial exposure to 100 cercariae after 6 months, while monkeys exposed to 600 cercariae show a profound response during the 4th month of infection.

Significance to Biomedical Research and the Program of the Institute: The regular production of hydrohephrosis in monkeys infected with S. haematobium opens the anatomical and functional study of these complications to free experimental approach for the first time. First steps will include a detailed study of the pathological lesions and of the quantitative parasitology (chiefly numbers of eggs in the tissues). The production of bladder cancers should allow us to follow the morphological and biochemical changes as they develop, and arrangements for collaborative studies to that end are in progress. Hydronephrosis and bladder cancer are common and serious sequellae of this infection in man in hyperendemic areas.

Comparison of S. japonicum infections in monkeys and chimpanzees will give considerable insight into the relation of host reaction to the eggs to the eventual development of pipestem fibrosis, as it already seems evident that some species of monkeys will not develop this lesion. Immune complexes would seem the most likely provacative agent in the observed glomerulonephritis, and the experimental situation favorable to demonstrating their importance. The chimpanzee-S. japonicum system is the only usable system which exists for the experimental study of pipestem fibrosis, the principal severe complication of S. mansoni and S. japonicum infections in man. We are therefore particularly concerned about finding an alternative experimental host, and in deriving the most information possible from infected chimpanzees.

Proposed course of the Project: In vitro correlates of delayed hypersensitivity are being followed in S. japonicum infected chimpanzees. The developing lesions in the kidneys and liver are being studied ultrastructurally and by immunofluorescence. Gastrointestinal protein loss will be studied in monkeys infected with S. mansoni and S. japonicum. Marked intestinal lesions are present, and hypoalbuminemia is frequent. The African green monkeys maintained on contract will be killed soon, nearly three years after the initial infection.

Honors and Awards:

Dr. Cheever served on the U.S. Parasitic Diseases Panel of the U.S.-Japan Cooperative Medical Science Program, on the Council of the American Society of Tropical Medicine and Hygiene and on the Editorial Board of its Journal.

He also was sent to Brazil as a consultant by the Pan American Health Organization.

Publications:

Sadun, E.H., von Lichtenberg, F., Cheever, A.W. and Erickson, D.G.: Schistosomiasis mansoni in the Chimpanzee: The natural history of chronic infections following single and multiple exposures. *Am. J. Trop. Med. Hyg.* 19: 258-277, 1970.

Sadun, E.H., von Lichtenberg, F., Cheever, A.W., Erickson, D.G. and Hickman, R.L.: Experimental infection with *Schistosoma haematobium* in chimpanzees: parasitologic, clinical, serologic and pathologic observations. *Am. J. Trop. Med. Hyg.* 19: 427-458, 1970.

Cheever, A.W.: Relative resistance of the eggs of *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* to digestion in potassium hydroxide. *Bull. Wld. Hlth. Organ.* 43: 601-603, 1970.

Cheever, A.W., Kirschstein, Ruth L. and Reardon, Lucy V.: *Schistosoma mansoni* infection of presumed natural origin in *Cercopithecus* monkeys from Tanzania and Ethiopia. *Bull. Wld. Hlth. Organ.* 42: 486-491, 1970.

Cheever, A.W. and Andrade, Z.A.: Comparison of pathological changes in the livers of subjects with compensated and decompensated hepatosplenic schistosomiasis. *Gaz. Med. Bahia.* 70: 67-74, 1970.

Cheever, A.W.: Analise quantitativo de esquistossomose mansoni. *Segundo simposio sobre esquistossomose*. pp. 86-100 and 117-118, 1970.

Cheever, A.W. and Anderson, L.A.: Rate of destruction of *Schistosoma mansoni* eggs in the tissues of mice. *Am. J. Trop. Med. Hyg.* 20: 62-68, 1971.

Cheever, A.W. and Powers, K.G.: Rate of destruction of *Schistosoma mansoni* eggs and adult worms in the tissues of rhesus monkeys. *Am. J. Trop. Med. Hyg.* 20: 69-76, 1971.

Andrade, Z.A. and Cheever, A.W.: Alterations of the intrahepatic vasculature in hepatosplenic schistosomiasis mansoni. *Am. J. Trop. Med. Hyg.* (In press).

Erickson, D.G., Lucia, H.L. von Lichgenberg, F., Cheever, A.W. and Sadun, E.H.: *Schistosoma haematobium* infections in five species of primates. *Exp. Parasitol.* (In press).

Kuntz, R.E., Myers, B.J. and Cheever, A.W.: *Schistosoma haematobium* infection in the opossum (*Didelphis marsupialis*): involvement of the urogenital system. *Bull. Wld. Hlth. Organ.* (In press).

Lee, H.G., Cheever, A.W. and Fairweather, W.R.: Influence of parasite strain on chemotherapy of murine schistosomiasis mansoni. Bull. Wld. Hlth. Organ. (In press).

Serial No. NIAID 121-I
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Serologic studies on filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: Anastasia Stanley

Cooperating Units: Major Dennis Johnsen, Department of Veterinary Medicine, United States Army Medical Component, South East Asia Treaty Organization.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	17/12			17/12
Professional	15/12			15/12
Other	2/12			2/12

Project Description:

Objectives: To study serologic changes in experimental and natural infections with various filariae, as well as *Toxocara canis*. Various technics with antigens from different stages of the life cycle of homologous or heterologous parasites are used in these studies.

Methods Employed: Indirect tanned-cell hemagglutination is used as the basic technic to evaluate antibody response. Diffusion in agar and immunoelectrophoresis are used as adjuncts in analysis of antigens; fluorescent antibody technics are used to demonstrate antigen-antibody complexes with living helminths.

Sera used in the above tests are from: (a) jirds or hamsters infected once or periodically with *Dipetalonema viteae*; (b) hamsters exposed from conception to microfilariae of *D. viteae*, infected once or periodically with third-stage larvae of the same parasite or transplanted with adult male and female D. viteae; (c) cats, monkeys and jirds injected once or periodically with third stage larvae of Brugia malayi; (d) cats and jirds injected once or periodically with third-stage larvae of B. pahangi; (e) dogs experimentally infected with Dirofilaria immitis and (f) mice and rabbits infected or reinfected with T. canis. Additionally, sera from patients known or suspected to harbor filariae are tested as required.

Antigens used for serologic tests are: (a) fractionated extracts of adult male and female D. immitis or T. canis; (b) metabolic products of third stage larvae of D. viteae, B. malayi or B. pahangi; (c) metabolic products of microfilariae of D. immitis, B. malayi or B. pahangi; (d) metabolic products of larvae of T. canis.

Major Findings: Evaluation of the specificity of the indirect tanned-cell hemagglutination test with extracts of adult D. immitis and sera from people that did not have filariasis indicated that an antibody titer of 1:160 was a good "cut off" titer between negatives and positives. Although only 7% of sera from 196 blood bank donors had antibody titers over 1:160; false positives were seen frequently as in sera from 4 out of 21 patients with syphilis, 3 out of 10 patients with lupus and 33 out of 145 patients with various types of malignancies. By contrast, sera from only 5 out of 45 patients with tropical pulmonary eosinophilia and 12 out of 40 American Indians had antibody titers less than 1:160. High anti-Dirofilaria antibody titers in sera from American Indians may reflect infection with filariae of animals.

To determine whether host-parasite relationships in hamsters exposed to microfilariae of D. viteae from ceonception differed from those noted in nonexposed hamsters, litters from hamsters mated when microfilaremia was first detected, and which were born with microfilaremia, were infected with third-stage larvae once or fortnightly; implanted with adult worms or maintained uninfected. Sera from these as well as untreated hamsters were collected at preset intervals and tested by IHA with extracts of adult D. immitis as well as metabolic products of larvae of D. viteae. The results observed with these sera were as follows: (a) with metabolic products of third-stage larvae of D. viteae antibody was not detected in sera from uninfected hamsters, hamsters that had microfilariae only or those in which adult worms were implanted. Low antibody titers were noted for a few weeks in sera from some of the hamsters given a single injection of third-stage l larvae whereas in sera from hamsters injected with third-stage larvae every other week high titers of antibody were detected. (b) With extracts of adult D. immitis antibody was detected in sera from all groups of hamsters, except those that were born without microfilaremia and were uninfected. The time of appearance of antibody and the titers noted were quite variable in all of the groups of hamsters born with microfilaremia. There was greater variability in the response noted from one litter of hamsters to another than there was within each litter, suggesting that the exposure to microfilariae in utero varied from one litter to another.

Sensitization of tanned erythrocytes by living infective larvae of B. malayi or of B. pahangi was found to require longer time periods than noted for larvae of D. viteae. However, the metabolic products of Brugia larvae which sensitize cells are much more stable than those of D. viteae and can be stored for short periods of time. Evaluation of both the antigens and the antibody systems in sera from animals infected with the homologous parasites will be continued.

Preliminary studies indicate that relatively few microfilariae (10,000-50,000) are required to sensitize tanned erythrocytes with metabolic products. Antibody detected by these cells appears to be specific, at least for this stage of the parasites.

Significance to Bio-medical Research and the Program of the Institute:

With few exceptions, serologic tests currently in use as aids in diagnosis of helminthic infections are believed to lack specificity or sensitivity. Inadequate experimental evaluation of the tests, in terms of relationship between parasite burdens, time during the infection or levels or kinds of antibodies present, has resulted in a lack of perspective to assess results obtained. Evaluation of serologic tests for nonpatent helminthiases such as infections with T. canis or filariae is particularly troublesome as there is little quantitative information on which to base conclusions. Antigens used in most current serological tests for filariasis are extracts of adult heartworm, but as evidenced by our results to date, it is clear that at least two types of antibody are detected in sera from animals infected with unrelated species of ailariae. These antibodies are not specifically related to larvae, adult worms or microfilariae, moreover they are detectable in sera from a significant number of people with unrelated diseases. By contrast, the results of tests with metabolic products of larvae or of microfilariae appear to be specific at least to the stage of the parasite and may more nearly reflect the biological relationship between host and parasite.

Proposed Course of the Project: Extracts of adult worms will continue to be used in an indirect hemagglutination test to provide comparative antibody titers with other antigenic substances. Emphasis will, however, be on the use of metabolic products of microfilariae, infective-stage larvae and possibly adult worms as antigens. Sera from animals that represent the various host-parasite situations that may be encountered in nature will be used. Additionally, it is hoped that sera from people with bancroftian filariasis as well as microfilariae will be available to extend these studies into filarial disease as seen in man.

Serial No. NIAID 121-J
1. Parasitic Diseases
2. Host-Parasite Relationships
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Host-parasite relationships in filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: Dr. Gabriel Schmunis, University of Buenos Aires, Buenos Aires, Argentina (Fogarty Fellowship); Dr. Ana Szarfman, University of Buenos Aires, Buenos Aires, Argentina (PAHO Fellowship); Dr. Stuart Beeber, Research Associate, PHS.

Cooperating Units: Dr. Thomas C. Orihel, Delta Primate Center, Covington, Louisiana; Dr. George S. Tulloch, San Antonio, Texas.

Man Years

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	31/12	0	4/12	35/12
Professional	21/12	0	4/12	25/12
Other	10/12	0	0	10/12

Project Description:

Objectives: To study the interaction between filariae and their vertebrate hosts. Additionally, other nematodes living in tissues would be studied. Hopefully, these studies would provide a better understanding of filariasis and nonpatent helminthiases in man.

Methods Employed: *Brugia malayi*, *B. pahangi*, *Dirofilaria immitis* and *Dipetalonema viteae*, as well as appropriate arthropod vectors, are maintained for experimental study. Cotton rats infected with *Litomosoides carinii*, blood and tissues from monkeys infected with *Dirofilaria corynodes* or *B. malayi* and adult *Toxocara canis* are received from collaborating laboratories.

Vertebrates are infected with filariae by injecting third-stage larvae recovered from an appropriate arthropod vector, by surgically implanting adult worms subcutaneously or by infusing washed microfilariae intravenously. Numbers of leucocytes, eosinophils and, when present, microfilariae are estimated at preset intervals. When vertebrate hosts are necropsied the adult worms that are recovered, as well as representative samples of tissue, are preserved for later study.

Mice or rabbits are infected with eggs of T. canis by gavage and for some experiments mice are injected intravenously with hatched larvae. Resistance to challenge infections with homologous or heterologous parasites is monitored during the first 24 hours, by counting larvae recovered from the tissues as well as by histologic reactions.

Major Findings:

A. Infections with filariae.

To determine whether in utero exposure to microfilariae, and other biproducts of a filarial infection, would modify the reaction of a host to later infection, hamsters infected with D. viteae were mated when microfilaremia was first detected. High microfilaremia was noted in one hamster sacrificed from each of the litters one to two days after birth. Hamsters born with microfilaremia, as well as hamsters of the same age born from uninfected mothers, were placed in groups in which each hamster would be infected once, reinfected at fortnightly intervals or receive two pairs of adult worms as implants. One group of hamsters born with microfilaremia and one born without were retained as controls of the various parameters measured.

Growth of the hamsters, as indicated by increase in weight, was identical in all test and control groups. However, growth of the hamsters and the concomitant increase in total blood volume, diluted the number of microfilariae per unit of blood. When the hamsters weighed 60-80 g 1 or 2 microfilariae only could be detected in 20 cmm samples of blood. Thereafter, despite concentration of 1 ml of blood (approx. 1/9 of the total blood volume) microfilariae were not detected. Thus, there was a suggestion that microfilariae gradually disappeared from the bloodstream. Throughout the first 13 weeks of observation eosinophilia in this group of hamsters was higher than in hamsters born without microfilaremia. Thereafter it was indistinguishable from eosinophilia in untreated hamsters. While eosinophilia increased after injection of infective-stage larvae into hamsters born with microfilaremia, this increase was not large enough to distinguish this group of hamsters from those born with microfilaremia but uninfected. Eosinophilia in hamsters that were reinfected fortnightly remained at a higher level than in those given a single injection of larvae. Again however, during the first 15 weeks of observation, eosinophilia in these animals could not be distinguished from eosinophilia in hamsters born with microfilaremia and retained uninfected. A significant increase in eosinophilia was noted within the first three weeks after adult worms were implanted. Thereafter eosinophilia decreased to a level comparable to that observed in hamsters born with microfilaremia. Eosinophilia in hamsters born without microfilaremia that received adult worms as implants was higher, though not significantly, than in hamsters born with microfilaremia. This eosinophilia remained high throughout the 9 week period of observation.

Although analysis of the data from this experiment is not complete it is clear that in utero exposure to microfilariae did not, (a) prolong the length of time microfilaremia was detectable, (b) modify microfilaremia in hamsters injected once or fortnightly with infective stage larvae or (c) result in more or less adult worms. On the other hand, as evidenced by eosinophilia and more rapid antibody response, the presence of microfilariae in the hamsters prior to birth modified subsequent host-parasite relationships.

In a second experiment to determine whether tolerance of microfilaremia of D. viteae in hamsters could be induced by injections of anti-hamster thymocyte sera (ATS), microfilaremia in hamsters injected with ATS disappeared as rapidly as in those injected with normal serum or noninjected hamsters. The results of this and the previous experiment suggest that clearance of microfilariae in the hamster-D. viteae system is not primarily an immunological response, it may simply be the result of an unfavorable environment. However, once the host has been sensitized by the microfilariae that die, subsequent microfilariae may be subject to immune reactions as well as the unfavorable environment.

Infections with B. pahangi and B. malayi have been established in the jird, Meriones unguiculatus. However, unlike infections with D. viteae in the same host, Dirofilaria immitis in dogs or Litomosoides carinii in cotton rats, large numbers of larvae - more than 50 - are required to establish microfilaremia. Moreover, microfilaremia increases in some of the jirds following reinfection. Jirds infected once only or reinfected at preset intervals are being monitored for changes in total white cells, eosinophils, weight and antibody titers.

B. Infections with Toxocara canis.

Results of previous studies on immunity to larvae of T. canis have not clearly demonstrated protection of a host to reinfection. As the intervals used in these studies were relatively short - maximum two weeks - our studies were designed to determine whether there were differences between the number of larvae recovered following inoculation of eggs at intervals of 5, 30 or 45 days. More than twice as many larvae were recovered from mice reinfected 5 days after first inoculation than from those reinfected 30 or 45 days after the first inoculation. There were no significant differences between the number of larvae recovered from mice reinfected after 30 or 45 days and mice that received a single inoculation. However, infection with large numbers of eggs (over 1,000) apparently overwhelmed the defense mechanisms of the host resulting in massive invasion of tissues.

Previous studies with Toxocara canis suggested that larvae rejected by an immune host died in liver or lung. To determine whether rejection might also occur in the intestine of mice infected 1, 2 or 4 times with the homologous parasite, groups of 5 mice each were sacrificed 4, 8 and 24 hours after challenge. Intestine and liver were removed from each of the mice. The intestine was washed to remove larvae that had not penetrated. Both organs were then digested and larvae counted. Maximum numbers of larvae were recovered 8 hours after infection at which time the intestine of previously uninfected mice contained approximately 45% of the inoculum. By contrast, tissues of mice infected once, twice or four times previously contained approximately 30%, 16% and 2% respectively of the number of larvae recovered from control mice. Prompt intense focalized infiltration of mononuclear leucocytes and late eosinophiles characterized the tissue response. From the above experiments it seems clear that, (1) development of immunity to T. canis requires more than one week, (2) that there is a threshold in number of larvae that will be rejected by the host beyond which the host is simply overwhelmed and (3) that with each successive infection the intestine becomes the barrier blocking penetration and further development of these larvae.

Significance to Biomedical Research and the Program of the Institute: While basic interactions of host and filariae are poorly understood, present availability of animals in which some of the human filariae - Brugia spp. - will develop provides an opportunity to study these relationships. Additionally, study of host-parasite relationships with several species of filariae, and of hosts, could indicate features common to the various infections which in turn would provide greater insight to the infections of man with one or more of the seven species regarded as human parasites. Studies to determine whether a host in which microfilaremia is present for weeks only could be modified by exposure to microfilariae in utero or by treatment with anti-thymocyte serum, indicated quite clearly that at least the primary disappearance of microfilariae was not an immunological event.

Studies on immunity to Toxocara canis indicated that while a host could be reinfected within a period of one or two weeks, the animal was resistant to further infection after that time, unless an overwhelming number of eggs were used as inoculum. The site at which larvae of T. canis were rejected was found to shift, after first challenge, from liver and lung to intestine. From these studies one may postulate that children infected with T. canis probably acquired their infection within relatively short periods of time and that once the larvae are encapsulated it is unlikely that additional worms will succeed in invading the host.

Proposed Course of the Project: In filariasis studies will be continued to determine, with various species of filariae, the sites at which microfilariae are destroyed, and the mechanisms by which they are destroyed. Additionally, sequential observations on various parameters of infections of jirds with Brugia spp. under conditions of single infection, reinfection, and transplantation will be done. With both the filariae and Toxocara canis attempts will be made to define more precisely the role of cell-mediated immunity in disappearance of microfilariae, or death and destruction of larvae.

Honors and Awards:

Dr. Pacheco was an invited discussant at the Colloquium on Filariasis of the Second International Congress of Parasitology, September 6 to 12, 1970 and a discussant at a Conference on Chemotherapy of Filariasis sponsored by the the U.S.-Japan Cooperative Medical Science Program. Dr. Pacheco gave lectures in Parasitology to first-year medical students at the University of Pittsburgh, School of Medicine and participated in meetings of the Tropical Medicine and Parasitology Study Section, DRG, NIH.

Publications:

Pacheco, G. and Tulloch, G. S.: Microfilariae of *Dirofilaria striata* in a dog. *J. Parasitol*. 56: 248, 1970.

Serial No. NIAID 122-G
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Entamoeba histolytica and other parasitic protozoa.

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Carl F. T. Mattern and Albert H. Gelderman

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	28/12		1/12	29/12
Professional	9/12		1/12	10/12
Other	19/12			19/12

Project Description:

Objectives: To develop and refine techniques for the axenic cultivation of Entamoeba histolytica, related amebae and other parasitic protozoa; to define their in vitro nutritional requirements, to study their metabolism, and mechanisms of pathogenicity. To ascertain factors involved in cyst production of Entamoeba. To study naturally occurring viruses of E. histolytica. To develop techniques for cryopreservation of parasitic protozoa, to study mechanisms involved in resistance of these organisms to freezing injury.

Methods Employed: Entamoebae or other parasitic protozoa freed of naturally associated microorganisms by use of microisolation procedures and/or a variety of antibiotics are established in monoxenic cultures with single species of trypanosomatids or bacteria, then adapted to grow under axenic conditions. In vitro growth is monitored through use of a Coulter electronic counter and/or standard counting chambers.

Lysed Crithidia sp. or factions obtained from these organisms by high-speed centrifugation are studied for their ability to induce cyst formation in E. invadens grown axenically.

Viruses of E. histolytica are characterized and studied using classical virological techniques.

Protozoa are suspended in a variety of cryopreservatives (glycerol, dimethylsulfoxide, glucose, etc.) and the effects of varying freezing and thawing rates on recovery of viable cells cooled to temperature of liquid nitrogen (-196°C) are studied.

Major Findings: An agent(s) has been isolated from several strains of axenically cultivated E. histolytica which meet the classical criteria for identification of viruses, i.e. passes through an ultra-filter, is transmissable in serial passage, has a fine structure akin to known viruses, and produces two well-defined cytopathological effects; one involving dissolution of the nucleus and eventual lysis of the cytoplasm, the other a simple cytolysis of the infected amebae. The virus(s) can be visualized under the electron microscope as polyhedral bodies (80 mμ) or thin frond-like filaments within the nucleus and later observed in the cytoplasm. In amebae undergoing adaptation to the rirgors of axenic growth viral particles are detected with difficulty in the homologous amebae due apparently to their low concentration. However, in continuous passage or upon isolation in heterologous strains of amebae, viruses are found in substantial number associated with cells showing cytopathological effects (Diamond and Mattern).

Studies on the factors involved in the induction of encystation in E. invadens in the presence of Crithidia sp. have continued. Lysed Crithidia treated with disodium ethylenediamine tetracetate (NaEDTA) to remove calcium and magnesium are incapable of inducing cyst formation. On the other hand, Crithidia treated with calcium chelated EDTA induced cyst formation almost equal to that produced in the presence of untreated controls. Addition of high concentrations of calcium and/or magnesium salts had no cyst inducing effect suggesting that other factors in addition to calcium are involved in cyst production in E. invadens (Diamond and Gelderman).

Axenic cultures of a strain of E. histolytica have been initiated with amebae grown in monoxenic cultures in association with a bacterium, Bacteroides symbiosus. This is the first demonstration that culture sources other than the ameba-trypanosomatid combinations originally employed can be used to supply amebae for axenic cultivation of E. histolytica. This had been ascertained earlier for E. terropinae and E. moshkovskii.

Increased yields of viable E. histolytica have been obtained after freeze-preservation in vapors of liquid nitrogen through use of 1) a cryopreservant mixture consisting of dimethylsulfoxide, glucose, serum and growth medium, and 2) a recovery mixture of growth medium containing high concentrations of serum (25 - 35%). Entamoeba histolytica has been recovered in a viable state after 10 years storage in the vapors of liquid nitrogen. This is the longest period recorded for the successful storage of any parasitic protozoan at this low temperature (-170°C).

Significance to Bio-medical Research and the Program of the Institute:

The discovery of virus in E. histolytica, the first to be clearly identified in any protozoan, opens a new field of research in protozoology, and provides virologists with a new system for investigation of a naturally occurring virus in a relatively simple animal cell. Moreover, the existence of the virus and its effect on the ameba will have to be taken into account in almost all future research in amebiasis.

The axenic cultures of E. histolytica continue to provide a basic research tool for studies of the metabolism, immunology, and molecular biology of this important parasite of man. The discovery of the viral agent of E. histolytica was to a great extent dependent on the use of axenic cultures.

Proposed Course of Project: Efforts will be made to further characterize the virus(s) of E. histolytica, and a search for virus in other parasitic protozoa will be initiated. Special emphasis will be given to development of an assay system for the amebal virus, since those of classical virology are not directly applicable. Further investigations will be made to pinpoint the role of calcium in cyst formation in Entamoeba and to elucidate other factors involved. Efforts to develop techniques for axenic cultivation of other parasitic amebae will continue, as well as efforts to improve the recovery of viable E. histolytica after storage at cryogenic temperatures.

Honors and Awards:

Chairman, American Type Culture Collection Advisory Committee to the Collection of Protozoa.

Member, Board of Trustees, American Type Culture Collection.

Member, WHO Expert Advisory Panel on Parasitic Diseases (General Parasitology).

Member, Executive Committee, Society of Protozoologists.

Member, Editorial Board, Cryobiology.

Invited Conference Leader, Third Seminar on Amebiasis, sponsored by the National University of Mexico and the Mexican Institute of Social Security, Mexico City, February 24-26, 1971.

Publications:

Diamond, L. S. and Bartgis, I. L.: Entamoeba moshkovskii: Axenic cultivation. Exper. Parasit. 28: 171-175, 1970.

Diamond, L. S. and Bartgis, I. L.: Axenic cultures for in vitro testing of drugs against Entamoeba histolytica. Prensa Med. Mexico (In Press).

Serial No. NIAID 122-I
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Quantitative and qualitative nucleotide studies of parasites

Previous Serial Number: Same

Principle Investigator: Albert H. Gelderman

Other Investigators: David B. Keister, Louis S. Diamond, and Milford N. Lunde

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	36/12			36/12
Professional	36/12			36/12
Other				

Project Description:

Objectives: (1) To characterize the nucleic acids of parasitic organisms in order to establish taxonomic relationships and the influence of evolutionary divergence. (2) To determine the nucleic acid synthesis of obligate intracellular parasites.

Methods Employed: Quantitative estimations of DNA are established by chemical analysis. DNA purification is accomplished with high ionic strength solutions in conjunction with hydroxyphatite chromatography. The base ratios of the nucleotides are determined by thermal stability and untracentrifugation. DNA reassociation kinetics are determined by using the Gilford recording spectrophotometer. DNA-DNA relatedness of different species is established using radioactive tracer labeled DNA, and the reassociated DNA is separated from non-reassociated DNA by hydroxyapatite chromatography.

Major Findings: Studies of the genetic potential and evolution of protozoal parasites are continuing. Our previous studies with the histolytica group of Entamoeba have indicated that these organisms may be separated into species on the basis of genome size, DNA complexity, and the percent of guanine and cytosine bases in the DNA. Further studies of the DNA from Entamoeba histolytica (HK-9) have suggested that this DNA may differ from bacterial or mammalian DNA in that the molecule contains a significant amount of glucose instead of ribose or that there are unusual bases. Attempts to isolate the bases have been unsuccessful but further work with ultracentrifugation is continuing.

Similar work is being done with the intracellular parasites. Since Toxoplasma gondii has life cycle stages characteristic of the coccidia, the DNA of T. gondii is being compared to that of other representative members of coccidia. DNA has successfully been isolated from oocysts of two species of Eimeria. The percent of guanine and cystosine in the DNA of these Eimeria organisms is significantly lower than that of T. gondii. Further studies of the DNA complexity and sequence homology of various members of coccidia are continuing.

Other studies of the nucleic acid metabolism of the obligate intracellular protozoa, T. gondii have shown that the parasite is not dependent on the host cell for either RNA or DNA synthesis. T. gondii requires preformed purine precursors for DNA synthesis but has the ability to synthesis pyrimidines from glucose or utilize preformed pyrimidines. The pyrimidine metabolism of T. gondii differs from that of the host cell in that high concentrations of preformed bases are required which would be inhibitory to the host cell.

Significance to Biomedical Research and the Program of the Institute:

DNA characterization appears to be a useful criteria for the taxonomic identification of protozoa. The studies of Entamoeba thus far have demonstrated that some non-pathogic amebae constitute different species. The studies have also suggested that there are different species of pathogenic Entamoeba. Studies of the intracellular coccidia parasites may demonstrate similar differences. The studies should also increase our understanding of the relationship of genetic potential to virulance and the evolution of parasites.

An understanding of the metabolic requirements of intracellular parasites will lead to new chemotherapy approaches. These studies also may explain the requirements for an obligate intracellular existence.

Proposed Course of the Project: Further studies of the DNAs from various isolates of E. histolytica will be done. Dr. Diamond has axenized several new isolates from Mexico and the United States. The DNA from these will be compared with those Asian isolates already studied. Studies are also continuing on the chemical nature of the Entamoeba DNA molecule.

Studies of the DNA from intracellular parasites will continue. The relationship of T. gondii to other coccidian organisms will be determined by

DNA characterization and sequence relatedness. The complexity of the DNA from virulent and avirulent intracellular parasites will be studied by reassociation kinetics.

Honors and Awards:

(1) Invited participant, Round Table Discussion on "Taxonomic Problems Relating to Malaria Parasites and Allied Organisms". Held at Fogarty International Center for Advanced Study in the Health Sciences (A.H. Gelderman)

(2) An invited lecture was presented by A.H. Gelderman to the class of Microbiology 200, graduate program NIH, entitled "Toxoplasmosis".

Publications:

Gelderman, A. H., Rake, A. V., Britten, R. J.: Transcription of nonrepeated DNA in neonatal and fetal mice. *Proc. Nat. Acad. Sci., U.S.A.* 68: 172-176, 1971.

Gelderman, A.H., Keister, D. B., Bartgis, I. L., and Diamond, L. S.: Characterization of the deoxyribonucleic acid of representative strains of *Entamoeba histolytica*, *E. histolytica*-like amebae, and *E. moshkovskii*. *J. Parasit.* (In Press).

Gelderman, A. H., Bartgis, I. L., Keister, D. B., and Diamond, L. S.: A comparison of genome sizes and thermal denaturation derived base composition of DNAs from several members of *Entamoeba* (histolytica group). *J. Parasit.* (In Press)

Siegal, S. E., Lunde, M. N., Gelderman, A. H., Halterman, R. H., Brown, J. A., Levine, A. S., and Graw, R. G., Jr.: Transmission of Toxoplasmosis by leukocyte transfusion. Blood 37: 388-394, 1971.

Perrotto, J., Keister, D. B., and Gelderman, A. H.: The Incorporation of Precursors into the DNA of *Toxoplasma gondii*. *J. Protozool.* (In Press).

Serial No. NIAID 122-J
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical Basis for Growth and Differentiation of Parasitic and Free-Living Nematodes

Previous Serial No.: None

Principal Investigator: Ruth Pertel

Other Investigators: Mary Vickers Burdett and Leland G. Dobbs

Cooperating Units: Lee Stevan, National Bureau of Standards
Dr. Stanley Falkow, Department of Microbiology
Georgetown University Medical School

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	22/12	1/12	10/12	33/12
Professional	22/12	1/12	10/12	33/12
Other				

Project Description:

Objectives: Parasitic nematodes that cause serious diseases in both man and domestic animals possess an interesting transitional point in their life cycle. They respond to specific, often host produced cues that not only seem to result in a behavioral change but also signal an initiation of the differentiation and development of the reproductive organs and germ cells. Biochemically, this is observed as an initiation of DNA (deoxyribonucleic acid) synthesis. This offers a well-defined system for a study of development and gene expression in a higher organism, evolutionarily and organizationally, than bacteria and viruses. In addition, an obvious dividend of this sort of study is the development of a means to control parasites.

A broad-based program has been initiated which utilizes biochemical, genetic and neurobiological techniques to investigate the life cycle of nematodes. The first phase is to study the environmental or host-produced cues that initiate infectivity or migration by parasitic and free-living nematodes. These are apparently modulated in the nervous system. The second phase is the study of the basic control mechanisms involved in the differentiation of the reproductive system occurring in the next phase of the life cycle.

Methods Employed: Free-living Caenorhabditis elegans var. Bristol and C. briggsae cultured as synchronous populations under axenic conditions (free of all living associates), are used as the initial test system. Chemotactic, motility and drug resistant mutants used to study the neuronal basis for the responses of nematodes to their environment are produced through selective use of methridine, Dupont Nematocide 1410, and piperazine.

Developmental mutants of C. elegans var. Bristol have been selected for properties that mimic the life cycle of many parasitic forms in which the third-stage molt form remains ensheathed (dauerlarva) and presumably is protected in the second stage molt for long periods of time, i.e. until its host for the reproductive stage appears. These developmental mutants are studied in regard to growth conditions, effects of the gaseous environment (CO_2 and O_2), population number, and age.

Nucleic Acid Methods:

Nematodes grown axenically are harvested, and disrupted either by sonication and treatment with proteolytic enzymes or in the French pressure cell at 500 psi. DNA obtained from the nematode homogenate by extracting with phenol or chloroform or by chromatography on hydroxyapatite is then characterized by thermal stability, analytical ultracentrifugation, and preparative centrifugation after specific labeling with the precursors and isolation of fractions for nucleotide analysis. Satellite DNAs are separated by preparative ultracentrifugation. Ribonucleic acids (ribosomal, transfer, etc.) are isolated by standard extraction with phenol and DNAase digestion. In vitro protein synthesizing techniques utilize the standard nucleic acid filter binding assays.

Major Findings:

A. Studies with mutants of C. elegans var. Bristol

It has been postulated (Rogers, W. P., Proc. Roy. Soc. Bri. 152: 367, 1960) that processess apparent in the internal control of the life cycle of the free-living nematodes have been lost during evolution to the parasitic habit. Therefore, the parasite would be dependent upon external agents, presumably produced by specific environments (hosts), to provide for the lost initiation factors for the control of specific events, e.g., the differentiation of the reproductive apparatus and modifications in the cuticle. If this is true, modifications of the genetic apparatus of the free-living nematodes by specific mutations should provide model systems of parasitic life cycles, notably those parasites postulated to be most closely related to the free-living Rhabditids, i.e., the Strongyloids. A mutant has been developed which manifests a life cycle dependency upon a change of medium after it has gone through a reproductive cycle and growth to the third stage. New medium is necessary to initiate another round of reproduction. The mutant demonstrates multifactor inheritance. In addition, clones have been isolated in which the early juvenile stages exhibit neurological disorders (trembling, semi-paralysis) that are not present in the adult stages (Pertel and Dobbs).

Nematode development is punctuated by four molts. Previous studies by other authors demonstrate that the molt process is only loosely linked to growth. However, control of the growth process is apparently linked to events of the third molt stage. Genetic analysis of several mutants in which growth (in terms of cytoplasmic accretion) has ceased at the time of the differentiation of the gonads reveals that these are single genes expressed as sex-linked recessives. Mutants at any other molt stage have not been found. This phenomenon cannot be overcome by increased supplementation of the medium.

Preservation of populations (genomes) utilized in genetic and biochemical manipulations in time is an important consideration in experimental controls. Since spontaneous mutation rate continues to act upon a population as long as it is reproducing, one of the only methods for maintaining a stable genome is cryo-preservation. A technique for the preservation in liquid nitrogen vapor of the mutants has been developed. Recovery of viable populations is high, approaching 100% in most cases, and progeny populations breed true according to our criteria.

B. Modulation of Environmental Stimuli by the Nervous System of Nematodes

The existence of a relatively simple link between the environment of a variety of nematodes and developmental events has been demonstrated previously in other laboratories. This link involves sensory receptors and neurosecretory cells. Very little has been reported concerning the nature of the neural transmitters in nematodes and their synthesis, storage and mechanism of action. The only previous information concerns evidence that the activating neuromuscular transmitter is acetylcholine and that the inhibitory transmitter is gamma-aminobutyric acid (GABA). The presence of acetylcholinesterase, the enzyme that hydrolyses the activating transmitter-acetylcholine, has been demonstrated in a number of parasitic nematodes. In at least two parasites, the enzyme activities have approached that of the eel electroplax, which is the tissue of choice to study the acetylcholine transmitter system. Therefore studies of nematode neurotransmitters would provide us with information concerning basic mechanisms of neurobiology pertinent to all forms with cholinergic transmission, information on the critical phases of free-living and parasitic nematode life cycles; and information which would provide a more rational approach to the mode of action of nematocides.

Assays for a number of enzymes which are present in the nematodes and are also present in the cells of the nervous system of mammals have been developed (Pertel, Gershon, and Nirenberg). Since entry into this laboratory in September, 1970, work has continued on these assays. Cholinergic components have been demonstrated. The possibility of an adrenergic system is highly probable on the basis of the presence of synthetic enzymes. Serotonin, histamine and glutamic acid decarboxylase (GABA synthetizing enzyme) are also present at high levels.

Several nematocides have been used in the acetylcholinesterase assay to differentiate between the true acetylcholinesterases and non-specific cholinesterases. The DuPont Nematocide 1410 is nearly as potent as the best inhibitor for the enzymes of eel and human acetylcholinesterase, though its mode of action prior to this finding was only postulated.

C. Nucleic Acids

The isolation and purification of deoxyribonucleic acid (DNA) from Caenorhabditis briggsae has been accomplished by hydroxyapatite chromatography. These are extremely active nucleases, hence extraction and purification must be performed rapidly. On the basis of thermal stability and analytical ultracentrifugation a number of peaks of DNA have been isolated. There are marked differences between the younger molt stages and the reproducing adults. A heavy satellite of DNA has been found (Pertel and Burdett).

Preparation of C. elegans var. Bristol wild type adult DNA reveals several satellite components that approximate those found in C. briggsae. These have been characterized by analytical ultracentrifugation and melting point determinations. Treatment of C. elegans immature stages with hydroxyurea (which specifically inhibits DNA synthesis) prevents the development of the gonads with the continuance of normal nonmitotic growth (Pertel and Dobbs).

Differences in the availability of the tRNAs for protein synthesis during development have been shown. It is postulated that control expressed at this level of protein synthesis (translational) may be responsible for some of the developmental controls of metazoans. An assay for the binding of leucine to its specific tRNA has been worked out for the nematode system and can be utilized to identify the transfer RNA gene products, as well as serve as a model for the conditions necessary for the other amino-acid tRNA charging systems. The nematode enzyme has marked differences in assay conditions from the mammalian enzymes in terms of temperature, ionic requirements, rates, etc. These may serve as the basis for the design of nematocides that will preferentially affect the nematode rather than the host (Burdett, Falkow, and Pertel).

Significance to Bio-medical Research and the Program of the Institute:

Parasitic nematode infections of man, domestic animals, and plants are protean in character and world-wide in distribution. While there exist many anti-helminthic drugs and preparations, little is known about the exact mechanism of action of these compounds, especially when the state of knowledge is compared with what is known about antimicrobial agents. Part of this lack of knowledge stems from an inadequate understanding of the basic developmental processes and basic metabolism of nematodes; there is every reason to believe that a better understanding of basic life processes will enable one to select with greater accuracy possible antihelminthic agents. What is known lends weight to this belief. For example, the characteristics of the neural transmitters, their related enzymes and the neuromuscular junction in nematodes are only now being elucidated. Yet piperazine, methrydine, DuPont 1410, tetrachlorethylene, all common effective nematocides said to act on the nervous system of nematodes have been developed randomly and little is known of their mechanism of action other than that paralysis results upon application.

The nematode system also offers a good study system for the understanding of the basic biology of differentiation and development. It is an in vivo system that uniquely separates the differentiation of one tissue, the gonad, from the differentiation of any other tissue. Most other systems where blast stages undergo differentiation in a well-controlled fashion are those in tissue culture, i.e. in vitro systems with their attendant problems. The information derived from these studies will also provide an understanding of the basic interactions attendant upon the development of the host-parasite relationship in nematodes.

Proposed Course of the Project: Work on the life-cycle model system developed with the mutants will be intensified to determine the specific environmental stimuli involved. The transduction of this environmental information by the nervous system of the nematode will be studied in relation to its effects upon the life cycle and development. The nucleic acid studies will concentrate on the description of the satellite DNAs and transfer RNAs and the elucidation of the differences apparent at the various stages in the life cycle. A variety of related parasitic forms (eg. Strongyles) will be subjected to similar investigations in parallel studies.

Honors and Awards:

Invited lectures were presented by Ruth Pertel to the Laboratory of Molecular Biology, NIAMD and to the Biophysics Group, Carnegie Institution of Washington, D.C.

Serial No. NIAID 123-B
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathological physiology and histochemistry of parasitic diseases.

Previous Serial Number: Same

Principal Investigator: Dr. Teresa I. Mercado

Cooperating Units: Laboratory of Microbiology, NIAID (NIAID-207)

Man Years

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To gain a better understanding by means of physiological and histochemical studies of the pathological physiology of parasitic diseases.

To elucidate factors that may complicate a parasitic infection and consequently modify the known pathological physiology of the host induced by the infection.

Methods Employed: Assays of calcium and of cytochrome oxidase activity are made on livers from mice infected with the Tulahuen strain of *Trypanosoma cruzi* employing atomic absorption spectroscopy, Warburg respirometry, Kjeldahl, and standard histochemical techniques.

The role of mycoplasma in the paralyzing syndrome of rats infected with *Plasmodium berghei* is studied by means of blood-passaging in splenectomized rats, animal inoculations, and using standard culture techniques.

Parasitemia levels are determined by hemocytometer counts and microscopic examination of blood smears stained with Giemsa.

Major Findings: Studies initiated last year to examine the possible influence of calcium on the diminished activity of succinate dehydrogenase and cytochrome oxidase previously observed in the livers of mice infected with Trypanosoma cruzi indicated a significant elevation of this cation. The observations completed this year show that after 6 to 8 days of infection and parasitemias ranging from 16 to 312 million trypanosomes per ml. of blood (average: 77 million) marked necrosis of the liver occurred which was accompanied by an accumulation of calcium equivalent to 42 percent increase over the normal level. It was also observed that animals infected for a longer period of 9 to 10 days exhibited a similar increase in calcium although their parasitemias were lower than 10 million. The apparent discrepancy between severity of infection, judged from the parasitemia leval, and the rise in calcium may be explained by the fact that the liver phase of this tissue-invading trypanosome is accompanied by fluctuations in parasitemia after the initial marked increase, usually observed on the sixth or seventh day, although marked damage to this organ resulting in necrosis and calcium accumulation appears to persist. Parallel histochemical studies revealed deposits of calcium which ranged from moderate to extensive in some livers but was negligible in others. It should be emphasized that in the histochemical assays random, not serial, sections were examined and the technique used detects exclusively the presence of insolable calcium salts. In the biochemical assays larger samples were analyzed and the method used detects ionized, unionized, and protein-bound calcium. The incorporation of calcium, in the amounts detected over the normal level, did not appear to influence the activity of cytochrome oxidase, the only enzyme system studied, when tested either biochemically or histochemically; indicating, as documented in a previous study, that the major part of the reduced oxidative activity is due to loss of mitochondria resulting from necrosis and not to calcium accumulation as has been reported by other investigators for certain hepatotoxins. Further studies have also shown that the plasma level of calcium of mice infected 6 to 8 days exhibiting parasitemias of 10 to 62 million was normal.

Studies with the paralyzing strain of Plasmodium berghei were continued in new attempts to culture the paralyzing agent. Two mycoplasma strains isolated last year did not produce paralysis and showed the characteristics of avian species, not of Mycoplasma neurolyticum, long suspected to be the agent involved in the paralyzing syndrome. For the new experiments we continued to use the strain after it was passaged serially in splenectomized rats, a technique developed last year which was found to enhance virulence. Since it appeared from many trials that the rat so modifies the infection as to render the paralyzing agent unculturable, it was decided to pass the strain from paralyzed rats to white mice in attempts to restore the optimal conditions necessary for cultivation of the organism. The white mouse is the natural host of M. neurolyticum. In the initial trials employing conventional mice paralysis was produced following an incubation period of 5 to 6 days and the animals exhibited the typical "rolling disease" syndrome characteristic of M. neurolyticum infections. Culture studies revealed further the presence of M. neurolyticum in the brain of the paralyzed mice. Although the statistical evidence gathered from conventional mice appears reasonably convincing, ultimate isolation from germfree mice would be preferred since, unlike conventional mice, these have been shown not to harbor this organism. Such studies are in progress.

Significance to Biomedical Research and the Program of the Institute: Basic cellular studies provide a means for understanding the physiological mechanisms involved in the response of the host to parasitic infections and may lead to the identification of specific parasite substances involved in pathogenesis. Histochemical studies aid in localizing the altered sites within tissues. Physiological studies quantitate the alteration of activity o observed histochemically and aid in defining further the physiological mechanisms that may be damaged.

The paralytic syndrome resulting from the association between a malarial infection and a mycoplasma points to the increasing importance of research related to the behavior of mixed infections. The significant changes observed following splenectomy indicate the need for continued studies to define the role of the spleen in the malaria-mycoplasma interaction.

Proposed Course of the Project: The calcium studies on *T. cruzi* infected livers have been completed and the data are being prepared for publication. Future biochemical and cytochemical studies will examine the respiration of mitochondria from liver and heart muscle, during infections with *T. cruzi*, as well as their calcium-binding properties.

Attempts to culture the paralyzing agent will be continued employing germfree mice. Further evidence linking *M. neurolyticum* with the paralyzing syndrome will be gathered employing passive immunization of rats with antiserum prepared in rabbits against the toxin of this organism.

Honors and Awards:

Appointed member of a Task Force (Agency of International Development) to prepare a report on the Second International Congress of Parasitology, Washington, D. C., September 6-12, 1970.

At the request of Dr. Carlos Diaz-Coller, Chief, Department of Scientific Communications, Pan American Sanitary Bureau, Regional Office of the World Health Organization, edited the text for a filmstrip to be sent to all schools in Latin America entitled: "Enfermedades Helminticas, Ascariasis y Tricuriasis."

Presented an invited seminar before the Mycoplasmologists Luncheon Club on February 9, 1971, entitled: "Suspected Association of mycoplasmas with a paralytic *Plasmodium berghei* syndrome in rats."

Serial No. NIAID 123-C
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

Previous Serial Number: Same

Principal Investigator: Eugene C. Weinbach

Other Investigators: James Dvorak, Louis Diamond

Cooperating Units: Medical Neurology Branch, NINDB (King Engel, Pieter Kark)
Metabolic Diseases Branch, NIAMD (Sidney Wolfe)
Metabolism Branch, NCI (Donald Tschudy)
Department of Anatomy, Johns Hopkins Medical School (Charles Hackenbrock)

Man Years

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	30/12			30/12
Professional	15/12			15/12
Other	15/12			15/12

Project Description:

Objectives: To conduct fundamental studies on the mechanism of aerobic energy metabolism in mammalian and in parasitic organisms. Specifically: (A) To continue studies of electron transport and oxidative phosphorylation in mammalian mitochondria, and (B) comparative studies with mitochondria from parasites.

Methods Employed: Animal tissues are fragmented by mechanical or enzymatic disruption of the cells and mitochondrial fractions isolated by differential centrifugation. Mitochondria also are isolated from human biopsy specimens. Similar procedures are used to isolate mitochondria from cestodes, the parasites principally used in these studies.

Entameba histolytica, strain HK-9, is cultivated axenically in Diamond's liquid medium, and the active trophozoites collected from 72-hour cultures by centrifugation.

Human platelets are isolated from freshly drawn whole blood by differential centrifugation.

Fibroblasts are grown from skin punch biopsies obtained from normal patients and from patients with acute, intermittent porphyria. The cells are harvested by trypsinization and centrifugation.

Ehrlich ascites tumor cells, maintained in mice, are isolated from the peritoneal fluid by differential centrifugation.

Cellular constituents are determined and characterized by appropriate enzymatic, chemical and physical methods. Manometric and polarographic techniques are used to measure rates of oxidation.

Major Findings:

A. Parasite Studies

1. *Cestode mitochondria*: Studies were continued on the role of non-heme iron in the respiratory chain of cestode mitochondria. Direct chemical analysis of the mitochondrial fractions of *Taenia taeniaeformis*, *Hymenolepis microstoma*, and *Hymenolepis diminuta* revealed that the ratio of non-heme to heme iron exceeds that found in mammalian mitochondria. Low temperature paramagnetic resonance studies, done in collaboration with Dr. H. Kon, NIAMD, demonstrated that when these cestode mitochondria were respiring on a-glycerophosphate, the characteristic non-heme iron signal developed at $g=1.94$. The signal was abolished in the presence of iron-chalating agents. These findings extend our previous work and now provide conclusive evidence that non-heme iron proteins not only are present in cestodes but are functional in their respiration.

Preliminary evidence has been adduced during the year that DT-diaphorase is active in both the soluble and particulate fractions of *T. taeniaeformis*. This enzyme shown for the first time to be present in parasites, may provide an important mechanism for the reoxidation of reduced pyridine nucleotides formed during glycolysis. This is of particular significance in regard to cestodes because of their limited repertoire of oxidative enzymes.

2. *Entamoeba histolytica*: Investigation of the aerobic metabolism of intact *E. histolytica* was continued in collaboration with Dr. L. Diamond. Use of a wide array of respiratory inhibitors and exogenous substrates has shown that the respiration of the parasite is not associated with a functional tricarboxylic acid cycle. The absence of mitochondria in this organism also supports this finding. Preliminary studies with three chemotherapeutic agents, in concentrations that suppress the growth of *E.*

histolytica in culture, disclosed that Parmomycin had no effect, Emetine inhibited, and Metranidazole enhanced the respiration of the freshly isolated trophozoites.

B. Mammalian studies

1. Role of non-heme iron in mitochondrial energy-conservation reactions: 2-Thenoyltrifluoroacetone (TTA) inhibits electron transport in cestode and mammalian mitochondria by chelating a non-heme iron moiety of the respiratory chain. As we reported previously, TTA also inhibits oxidative phosphorylation and related energy-dependent reactions in mammalian mitochondria. In our preliminary studies, the inhibition appeared to be substrate specific in that true uncoupling of oxidative phosphorylation occurred only with NAD^+-linked substrates. More detailed study of this phenomenon has shown that both Site 1 (NADH to cytochrome b span of the respiratory chain) and Site 3 (cytochrome c to O_2) phosphorylations are uncoupled by TTA. The enzymes associated with Site 2 (cytochromes b to c) phosphorylation were found to have such strong affinity for TTA, that is is impossible to determine the effect of this compound on oxidative phosphorylation at this site. Observation completed this year also show that a good correlation exists between the ability of given compound to bind non-heme iron and its ability to uncouple oxidative phosphorylation. These findings indicate that non-heme iron, in addition to serving as prothetic groups for electron transfer proteins of the respiratory chain, also participates in the energy-conservation reactions of the mitochondrion.

2. Oxidative phosphorylation and ultrastructural changes of mitochondria *in vivo*: In collaboration with Dr. C. Hackenbrock, Johns Hopkins School of Medicine, a study was initiated of the ultrastructure of mitochondria as it relates to energy metabolism of the intact cell. Oxidative phosphorylation was induced in intact Ehrlich ascites tumor cells by rapidly generating intracellular ADP from endogenous ATP by addition of 2-deoxyglucose (2-DOG). That oxidative phosphorylation occurred under these conditions was confirmed by analysis of intracellular adenine nucleotides and inorganic phosphate. Coincident microsamples of cells rapidly fixed for electron microscopy revealed that, in addition to oxidative phosphorylation, an ultrastructural change in the mitochondria had occurred, from the "orthodox" to the "condensed" configuration. Neither oxidative phosphorylation nor ultrastructural changes were observed in mitochondria in the intact cell in the presence of uncouplers of oxidative phosphorylation. These and other parameters of the ultrastructural transitions which were studied show that morphological changes in mitochondria are linked to induced oxidative phosphorylation induced in the intact ascites tumor cell. Such studies serve as an important model for other cell types.

C. Human Studies

1. Biochemical changes in skeletal muscle mitochondria from myopathic patients: The collaborative study with Drs. K. Engel and P. Kark, NINDS, designed to determine whether mitochondrial abnormalities provide a

biochemical basis for certain myopathies, was continued. Mitochondria were isolated from skeletal muscle biopsies obtained from two patients diagnosed as having a ragged-red fiber myopathy. Respiratory rates and degrees of energy coupling were tested under a wide variety of conditions. Means were devised of comparing the data from patient to patient which circumvented the previous problem of the contamination of mitochondrial fractions with non mitochondrial protein. The most striking abnormality found was a low Q_{O_2} with exogenous NADH, when respiration proceeded via the mitochondrial pathway linked to energy conservation, but not when the extramitochondrial pathway was utilized. The oxidation of NAD-linked substrates also was diminished in mitochondria from myopathic patients compared to normal controls, although not to the same extent as observed with NADh. The P:0 ratios and respiratory control ratios were essentially normal in all of the mitochondrial preparations tested. More data are needed before definitive conclusions can be made regarding mitochondrial defects as a basis for human muscle diseases.

2. Respiration of human platelets: In conjunction with Dr. Sidney Wolfe, NIAMD, a study was initiated this year to ascertain if aerobic energy-conservation processes are casually related to the clotting mechanism. Platelets suspended in buffered salt media readily consume oxygen, and upon the addition of thrombin a rapid burst of oxygen uptake ensues. This finding, now firmly established, settles a long standing controversy regarding platelet respiration. Use of inhibitors, known to selectively affect mitochondrial respiration, disclosed that both the basal rate of respiration, and the thrombin-induced burst, were related to energy conservation reactions of the platelet, presumably at the mitochondrial locus. Thrombin also releases calcium and adenine nucleotides ("release reaction") prior to the aggregation of platelets. Differential sensitivity of the basal rate of respiration and that of the thrombin-induced burst to selective inhibitors provided evidence that the respiratory substrates differ in the two oxidative processes. The respiratory burst and the "release reaction" was completely inhibited by prostagladin, whereas the basal rate was unaffected. The most significant finding of this study is that agents known to affect energy metabolism of isolated animal mitochondria can be used effectively to "titrate" platelet mitochondria in vivo, and thereby aid in elucidating an important mechanism in human physiology.

3. Biochemical studies of fibroblasts from patients with acute, intermittent porphyria: This study was initiated late in the year in collaboration with Dr. D. Tschudy, NCI. The most apparent biochemical characteristic of clinical porphyrias is the increased excretion of porphyrin or porphyrin precursors. The normal level of these compounds is believed to be controlled by the mitochondrial ratio of NAD/NADH. Work by others has demonstrated that drugs used to induce porphyria in experimental animals inhibit oxidation of NADH in liver mitochondria. Our preliminary studies of fibroblasts derived from patients with acute, intermittent porphyria show that these cells also have a defect in mitochondrial oxidation of NADH. Fibroblast respiration is sensitive to rotenone and a number of agents used to induce porphyria. Although our findings are preliminary, they are of considerable interest because they may lead to the first unambiguous demonstration of a genetically-linked mitochondrial disease.

Significance to Biomedical Research and to the Program of the Institute: This investigation, because of its fundamental nature in attempting to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious importance for biomedical research in general. This is seen in the several collaborative projects related to clinical studies. This investigation also provides a basis for understanding bioenergetic problems associated with parasitism. Studies of energy metabolism in parasites, apart from their intrinsic and comparative interest, contribute toward a more rational understanding of the host-parasite relationship, and may provide leads for chemotherapeutic exploitation.

Proposed Course of the Project: The parasite studies will be continued with emphasis upon energy conservation reactions in cestodes. The first phase of the study of aerobic metabolism in Entamoeba histolytica has been concluded. Future studies are planned to localize the intracellular site and further characterize the respiratory pathway in this parasite.

The mammalian studies will be pursued in regard to the role of non-heme iron proteins in oxidative phosphorylation. The collaborative projects involving biochemical studies of human platelets, fibroblasts, and skeletal muscle mitochondria will be continued. The study of oxidative phosphorylation in relation to ultrastructural changes of mitochondria in vivo has been concluded.

Honors and Awards:

An invited lecture was presented by E.C. Weinbach to the graduate students of Catholic University on January 25 entitled: "Mitochondrial Research Today."

E.C. Weinbach and L. Diamond presented lecturers to the medical and graduate students of Johns Hopkins University, School of Public Health and Hygiene, on February 4, and May 18 on the axenic cultivation and aerobic metabolism of E. histolytica.

E.C. Weinbach was invited to participate in the International Symposium on The Biochemistry and Biophysics of Mitochondrial Membranes, Bressanne-Padova, Italy, June 26-29.

Publications:

Hackenbrock, C.R., Rehn, T.G., Gamble, Jr., J.L., Weinbach, E.C., and Lemasters, J.J.: Ultrastructural transformation in the mitochondrion: Its relationship to the energy state of the mitochondrion and the energy state of the cell. Proceedings of Colloqium on Bioenergetics: Energy Transduction and Photo-synthesis, Pugnochioso, Italy (BBA Library Series, Elsevier Publishing Co., Amsterdam, London, New York, (in press).

Hackenbrock, C.R., Rehn, T.G., Weinbach, E.C., and Lemasters, J.J.: Oxidative phosphorylation and ultrastructural transformation in mitochondria in the intact ascites tumor cell. J. Cell Biol. (In press).

Serial No. NIAID 123-E
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biophysical Parasitology

Previous Serial No.: Same

Principal Investigator: James A. Dvorak

Other Investigators: Thomas P. Hyde and Gabriel A. Schmunis (Guest Worker)

Cooperating Units: Biomedical Engineering and Instrumentation Branch,
Division of Research Services, NIH

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	32/12			32/12
Professional	23/12			23/12
Other	9/12			9/12

Project Description:

Objectives: To utilize biophysical techniques for the study of parasitological problems of medical importance. Major emphasis has been directed toward a better understanding of the host-parasite interactions in Chagas' disease. The approach is two-fold: (1) to obtain clear understanding of the interaction at the cellular and subcellular level of *Trypanosoma cruzi* with vertebrate cells and (2) to relate this information to what is observed in a population of infected cells *in vitro* and, hopefully, the general course of events *in vivo*.

Methods Employed:

(1) *In vitro* cultivation

A controlled-environment culture system has been designed and constructed which fullfills the exacting physiological and optical requirements for quantitative and qualitative analysis of cellular and subcellular events. This system has been in near-continuous use for almost one year.

Techniques have been developed whereby large populations of parasitized cells can be analyzed. These techniques, combined with a priori information of cellular and subcellular events, has resulted in the formulation of general mathematical equations which describe host-parasite interactions within a population and predict the course of subsequent events. Although this approach has been restricted to a study of the interaction of T. cruzi with a population of host cells, preliminary calculations indicate that this method of analysis may be generally applicable to a large variety of host-parasite interactions.

(2) Quantitative microscopy

Time-lapse cinemicrography is used to determine (1) the exact time course of the intracellular cycle of T. cruzi, (2) differences in the intracellular cycle of T. cruzi as a function of known mediating parameters and (3) photodocumentation of phenomena being analyzed by quantitative procedures.

Interference microscopy is employed to quantify changes in mass/volume relationships in vertebrate cells infected with T. cruzi. Analog data is converted to a digital format, interfaced with a digital time base and recorded on punch tape for computer analysis.

Polarizing microscopy is used to determine (1) the molecular organization of anisotropic subcellular organelles present in a parasite, and changes in molecular organization as a function of the developmental stage of the parasite, (2) molecular changes in anisotropic structures of a parasitized host cell.

Major Findings:

(1) The interaction of T. cruzi with unstimulated mouse peritoneal macrophages

The controlled-environment culture system was used to continuously study the interaction of unstimulated BALB/cAnN mouse peritoneal macrophages with trypomastigotes and epimastigotes of the Ernestina strain of T. cruzi.

The invasion of macrophages by motile forms of T. cruzi is an active process on the part of the parasite. Trypomastigotes enter macrophages anterior-end first; epimastigotes enter posterior-end first. Both trypomastigotes and epimastigotes were destroyed by macrophages. The time required by macrophages to destroy parasites appears to be a function of the age and physiologic state of development of the parasite. Reproduction of parasites within macrophages was never observed.

In vivo experiments demonstrated that BALB/cAnN mice can be infected with the Ernestina strain of T. cruzi. Thus, if in vitro observations reflect the true in vivo response, then parasites entering macrophages probably are eliminated as a threat to vulnerable host cells. Such a response could be of particular significance to the host at the site of entry of the parasite.

The ability to successfully infect BALB/cAnN mice and demonstrate parasitemia, however, indicates that some trypomastigotes apparently reach susceptible cells and are capable of reproduction and development in this host, presumably mainly in striated muscle.

It is conceivable that the interaction observed is unique to the Ernestina strain of T. cruzi and BALB/cAnN macrophages or, at best, "myotropic" strains of the parasite and macrophages from selected sources. The interaction between a highly adapted "reticulotropic" strain of the parasite and macrophages may be entirely different. Verification of such a difference might help to explain the significantly variable course of Chagas' disease in nature.

(2) The interaction of T. cruzi with primary bovine embryo muscle cells

Observations that the Ernestina strain of T. cruzi invades and reproduces within primary bovine embryo muscle cells provides an excellent system to study the general course of events which occur during the intracellular cycle. The cycle can be divided into five major phases: (1) penetration of the host cell by trypomastigotes, (2) "reorganization" of the trypomastigote to the amastigote before reproduction begins (there is a 40-80 hour lag period), (3) Reproduction of the amastigotes with doubling times of about 12-24 hours, (4) Differentiation of amastigotes to trypomastigotes and (5) Escape of trypomastigotes from the host cell.

These phases appear to be the most likely biological "pressure points" for attempts at interdiction of the cycle.

(3) Polarizing microscopy

The kinetoplast of T. cruzi is highly anisotropic. The coefficient of birefringence is approximately 2.2×10^{-2} and the sign is negative. This is representative of a highly oriented "paracrystalline" DNA structure.

Significance to Bio-medical Research and the Program of the Institute:

(1) The study of host-parasite interactions at the cellular and subcellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite. In order to unamiguously interpret this interaction, it is necessary to observe it in its entirety under highly controlled and reproducible conditions. The system we are using allows us to accomplish this. Furthermore, the ability to almost instantly stop the interaction at any time and examine it by other physical means may allow us to obtain a better understanding of observed phenomena. This information should be of significant value in the understanding of parasitic diseases in general.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment culture system developed with this program has stimulated a great deal of interest among investigators from other research areas. The culture system is commercially produced, and is used in other laboratories for studies of virus-host cell interactions and lymphocyte transformation studies.

Proposed Course of the Project: Research will continue in an attempt to further our understanding of the interaction of *T. cruzi* with vertebrate host cells *in vitro* and relate this information to the course of Chagas' disease in nature. Although the basis of this research remains dependent upon the newly developed culture system, it is hoped that this base can be expanded to include classical biochemical analyses of synchronized events and ultrastructure studies.

Honors and Awards: None

Publications: None

Serial No. NIAID 125-A
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Toxoplasmosis

Previous Serial Number: None

Principal Investigator: Harley G. Sheffield

Other Investigators: Marjorie L. Melton

Cooperating Units: National Animal Parasite Laboratory, USDA, Beltsville, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	16/12			16/12
Professional	16/12			16/12
Other				

Project Description:

Objectives: The study of Toxoplasma gondii infections in cats to compare with the course of the disease in man; to determine the stages and sites of development of the parasite in the cat; to investigate the possibility of other animals serving as natural hosts; to determine possible mechanisms by which oocyst infections can be acquired by man and animals.

Methods Employed: Cats are infected by feeding them cysts in mouse brain or oocysts isolated from feces of infected cats. Oocysts are collected by zinc sulfate or sucrose flotation. Infectivity of concentrated oocysts is determined by feeding them to mice and subsequent checking for organisms or anti-toxoplasma antibodies. Tissues from infected animals are used for histological or ultrastructural studies. Cultured cells are used for in vitro studies of development of sporozoites which have been artificially excysted from oocysts and inoculated into the cultures.

Major Findings: Identification of schizonts, macrogametocytes, microgametocytes, and microgametes of *Toxoplasma gondii* in cat intestinal epithelium has been confirmed. Cesarean delivered kittens reared in isolation were used to eliminate possible confusion of *T. gondii* with the normal coccidia of cats. Tissues obtained from these kittens were also used for electron microscope studies (See 125-I). In cyst-induced infections organisms were seen dividing by schizogony in the intestinal epithelium and by endodyogeny in subepithelial tissues. The latter situation is apparently comparable to the course of infection in other animals as well as man. Cats fed occysts also have both an intestinal and an extraintestinal phase. The latter has been demonstrated by the finding, through subinoculation techniques, of parasites in liver, lung and spleen 4 days after oocyst infection.

Development of infection after oocyst ingestion was studied in mice by taking blood samples at 15 min intervals for 6 hrs after inoculation and injecting them into uninfected mice. No parasites could be isolated. Intestine was reserved for histological study which is in progress. The possibility of migration by the lymphatic route is also being studied. Data regarding possible development through a portion of the life cycle is being sought.

The epidemiological role of the cat in toxoplasmosis was studied by determining reinfection rates. Cats infected with cysts could not be reinfected. Three of 5 cats infected with oocysts became reinfected when fed cysts. Two other cats, initially fed oocysts, did not become reinfected when challenged with oocysts. All cats which became reinfected had low antibody titers when challenged. This data indicates that cats ingesting cysts in nature probably excrete oocysts only once whereas those becoming infected by oocysts are able to be reinfected and again be a source of infectious oocysts.

In vitro cultivation has been continued using primary cat kidney cell cultures. Sporozoite induced infections develop by endodyogeny but when cultures are maintained for 2 to 3 weeks numerous cysts are present as well as proliferating forms. The mechanism of cyst formation is being studied using this model system. As yet no development similar to that seen in the cat intestine has been noted. To provide a better environment for such development, use of organ cultures of intestinal tissue is being initiated. Results of inoculation of parasites into these cultures are not yet available.

Studies on the mechanism of cell penetration by *Toxoplasma gondii*, *Besnoitia jellisoni*, and *Sarcocystis* sp. are being carried out in collaboration with the National Animal Parasite Laboratory, USDA. Experiments with quinine indicate that penetration of these parasites is inhibited in presence of the drug in a similar manner to that reported for *Eimeria* sp. This finding supports fine structure data which indicate that they all have a similar method of cell invasion.

Significance to Bio-medical Research and the Program of the Institute:

The identification of the cat as the natural host for *T. gondii* provides a new insight into the epidemiology of toxoplasmosis and could explain the wide-spread existence of anti-toxoplasma antibodies in man and animals. In addition, the linking of *T. gondii*, whose classification has been uncertain for many years, to the coccidia provides the investigator with many model systems which could be used for comparison purposes in the study of *T. gondii* in normal and abnormal hosts. The cat's role as a reservoir animal may be significant in the cosmopolitan distribution of the disease. Information on the immunological aspects of the disease in cats would show the possibility of reinfection and may provide new leads relative to the cat being an important reservoir of the organism. Elucidation of the various stages of the life cycle in the cat would contribute to a better understanding of the disease in man. Basic information on the biology of the organism would show the relationship of *T. gondii* to other protozoa and possibly give some explanation for the parasite's unusual lack of host specificity and unique means of multiplication.

Proposed Course of Project: Emphasis will be placed on studies of the course of oocyst infections in cats including development through asexual and sexual stages. These stages will also be studied *in vitro* using both cell and organ cultures. The immunological aspects of the disease in cats will be studied to give additional information on reinfection relative to the cat's being a reservoir of the organism. Studies on related organisms such as *Besnoitia jellisoni* and *Isospora* sp. will be continued.

Honors and Awards:

Invited lecturer on the biology of *Toxoplasma gondii*, NIH Graduate School.

Publications: None

Serial No. NIAID 125-I
1. Parasitic Diseases
2. Cell Biology & Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electron microscope studies on parasites

Previous Serial Number: Same

Principle Investigator: Harley G. Sheffield

Other Investigators: None

Cooperating Units: Imperial College Field Station, Ascot, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	20/12			20/12
Professional	8/12			8/12
Other	12/12			12/12

Project Description:

Objectives: To determine the fine structure of various parasites in relation to their taxonomy, life cycles, reproduction and other physiological functions.

Methods Employed: Parasites are obtained from their host animals or culture at the developmental stage desired or after appropriate experimental procedures. The parasite tissue is processed, sectioned and then studies with the electron microscope and/or cytochemical methods.

Major Findings:

In vivo studies:

1.) Kittens delivered by cesarean section were infected with mouse brain containing cysts of *Toxoplasma gondii*. Heavy infections were produced and parasites were found at all levels of the small intestine. Schizogonic stages seen in these animals were identical to those reported previously but without proof of being *T. gondii*. All stages of a typical coccidian life cycle have been observed. These are trophozoites, schizonts, merozoites, micro- and macrogametocytes, microgametes and oocysts. No differences in general structure from that of other coccidia have been noted. Studies of details of differentiation and transformation from one stage to another are now in progress. Growth and division by endodyogeny has been noted in the subepithelial tissues of the intestine. This type of development is comparable with that seen in most animals and man.

2.) Studies on the penetration of sporozoites of *Lankesteria culicis* into the midgut epithelium of *Aedes aegypti* have indicated that the plasma membrane of the gut cell is altered and penetration is probably facilitated by the discharge of materials from the flask-shaped organelle in the anterior of the sporozoite. This study is being continued to include intracellular development of the trophozoite and the changes occurring prior to the organism leaving the cell and becoming a lumen parasite. These studies are being carried out with Professor P.C.C. Garnham, Imperial College Field Station, Ascot, England.

3.) Morphological studies of the bursa and the dorsal vaginal valve of *Aedes aegypti* mosquitoes have been completed. This work was done in collaboration with Dr. Jack Jones, University of Maryland.

In vitro studies:

1.) For comparison with the sporozoites of *T. gondii*, those of *Isospora canis* have been obtained and studied. Lack of sufficient material has prevented a complete description but a general similarity has been noted. One difference is the presence of a refractile body in *I. canis*. This body has a very ordered structurethat is also different from that reported in other coccidia.

2.) The fine structure of cysts developing in primary cat kidney cell cultures has been studied. The structure of the cyst wall is identical to that seen *in vivo*. Extensive development of host cell rough surfaced endoplasmic reticulum has been observed around the periphery of the cyst. The dense material contained within the cisternae of this ER may indicate a reaction to the parasite and could be instrumental in the formation of the cyst wall. (See 125-A)

Significance to Biomedical Research and the Program of the Institute:

The finding of structural similarities between the asexual and sexual stages of *T. gondii* and those of other coccidian organisms confirms the relation of *T. gondii* to the coccidia and probably to the genus *Isospora*. Such confirmation provides new leads to the study of the biology and epidemiology of *T. gondii*. Study of the differences of development in the natural host compared to that in man and other animals could provide insight into the mechanism of host specificity. The use of *in vitro* techniques will facilitate study of these differences. Study of related organisms will provide additional information which should allow a better understanding of the structure of *T. gondii* and its relation to the functioning of the organism.

Proposed Course of the Project: Studies on the endogenous stages of *T. gondii* in cat intestinal epithelium will be continued. Attempts will be made to determine the mechanism of formation of both asexual and sexual stages. The method of oocyst wall formation will be studied and compared with that known for other coccidia. Means of penetration of sporozoites and merozoites will be investigated both *in vivo* and *in vitro* and compared with those found in related organisms. A minor portion of the time will be allotted to assisting other investigators when their needs for electron microscopy arise.

Honors and Awards:

Member, Agency for International Development Task Force, Second International Congress of Parasitology.

Member, Editorial Board, Journal of Protozoology.

Assistant Editor, Journal of Parasitology.

Editor, Proceedings of the Helminthological Society of Washington.

Publications:

Jones, J. C. and Sheffield, H. G.: The fine structure of the seminal bursa of Aedes aegypti (Linnaeus). *Mosquito News* 30: 270-271, 1970.

Jones, J. C. and Sheffield, H. G.: The fine structure of the dorsal vaginal valve of *Aedes aegypti* (Linnaeus). *Mosquito News* 30: 650-652, 1970.

Sheffield, H. G.: Schizogony in *Toxoplasma gondii*: An electron microscope study. *Proc.Helm. Soc. Wash*. 37: 237-242, 1970.

Sheffield, H. G., Garnham, P. C. C., and Shiroishi, T.: The fine structure of the sporozoites of *Lankesteria culicis*. *J. Protozool*. 18: 98-105, 1971.

Serial No. NIAID 125-K
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Amebiasis studies in India

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Milford N. Lunde

Cooperating Units: Dr. Om Prakash, All-India Institute of Medical Sciences, New Delhi, India (PL-480 Project, Agreement No. 01066-1)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	2/12		1/12	3/12
Professional	2/12		1/12	3/12
Other				

Project Description:

Objectives: To evaluate an intradermal test for amebiasis using an antigen, histolyticin, from axenically cultivated *Entamoeba histolytica*; to determine the usefulness of the indirect hemagglutination test in assessing the effectiveness of drug therapy in amebiasis; to obtain data on the epidemiological and pathological significance of the *E. histolytica*-like Laredo ameba; to study the pathobiology of the clinical manifestations of chronic intestinal amebiasis and amebic hepatitis, and the relation of amebic infection and non-specific ulcerative colitis.

Methods Employed: Clinical evaluation, stool examinations, hemagglutination and agar-gel serology, skin testing, Tracerlab tracer scanner (Model Sc-47), stool, liver and pleural exudate culture.

Major Findings: One hundred and forty two individuals, evaluated clinically and parasitology for amebic disease, were skin tested intradermally with histolyticin. Serum drawn at the same time was tested for hemagglutinating antibodies by means of the IHA test. One hundred of these individuals assessed clinically to be suffering from disease were classified as follows: Liver abscess - 19 cases, non-suppurative hepatitis - 26, dysentery - 8, non-dysenteric colitis - 46. The remaining 42, found to be free of clinical amebic disease and *E. histolytica* were classified as controls. *Entamoeba histolytica* was found in the stools or sigmoidoscopic material of all dysenteric and non-dysenteric colitis cases.

Sixteen of the 19 (84.2%) liver abscess cases were positive by the ID test as aginst 17/19 (89.5%) by IHA. The results of testing the other groups were: Non-suppurative hepatic, ID - 12/26 (46.1%): IHA - 16/26 (61.5%), dysentery, ID - 7/9 (77.7%): IHA - 8/9 (88.9%), non-dysenteric colitis, ID - 5/46 (10.8%): IHA - 8/46 (17.4%), controls, ID - 6/42 (14.2%): IHA - 5/42 (11.9%). Immediate skin reactions (15-60 min) were noted in 80.5% of the positives, dealyed reactions (24 hr) in 6.5%, and both immediate and delayed types in 13.0%, no side reactions were noted in any of the 142 individuals tested. The study of Kirkpatrick, Lunde and Diamond (see Serial Number NIAID 33-(c)) on healthy volunteers from non-endemic areas in the United States suggest that a high proportion of the positive cases in the India control group reflects previous amebic infection.

Tonicity studies on 10 strains of *E. histolytica* isolated in culture from infected individuals failed to show the presence of *E. histolytica*-like amebae in the sample.

Significance to Bio-medical Research and the Program of the Institute:

The data on the intradermal skin test with histolyticin, though meager and of a preliminary nature, does indicate that this test will have real value in the diagnosis of amebic disease.

Proposed Course of the Project: Continued efforts will be made to carry out the stated objectives of the project. Emphasis will be placed on an in-depth evaluation of the ID skin test.

Honors and Awards: None

Publications: None

Serial No. NIAID 125-P
1. Parasitic Diseases
2. Cell Biology & Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunological studies on toxoplasmosis and other parasitic diseases

Previous Serial Number: Same

Principal Investigator: Milford N. Lunde

Other Investigators: Louis S. Diamond, Albert H. Gelderman, Kendall G. Powers, Philip R.B. McMaster and John Finerty

Cooperating Units: Laboratory of Clinical Investigation, Laboratory of Microbial Immunity, NIAID
Medicine Branch NCI, NIH Blood Bank

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	19/12			19/12
Professional	14/12			14/12
Other	5/12			5/12

Project Description:

Objectives: To differentiate and characterize the immune response in acute, chronic and reactivated latent toxoplasmosis and to evaluate diagnostic methods for detecting reactivation of the disease; to investigate the significance of toxoplasmosis complicating leukemia and the importance of blood transfusions in the transmission of toxoplasmosis; to determine the interrelationship of tumor viruses and protozoan infection in experimental hosts; to evaluate the skin test as an aid in the diagnosis of amoebiasis; to characterize the antigens of different strains of *Entamoeba histolytica* and *E. histolytica*-like entamoebae.

Methods Employed: The dye, hemagglutination, (IHA), complement fixation, agar-gel diffusion, and indirect fluorescent antibody (IFAT) tests are used to determine serum antibody. The presence of organisms is determined by inoculation of blood and other tissues into mice.

In chemotherapy studies mice are inoculated intraperitoneally with toxoplasma and treated per os using various schedules and dosages. The mice are bled for serology and immunoglobulin measurements. They are then killed for parasite isolation 42 days post infection. Surviving mice are challenged with toxoplasma to see if they are immune. If the treated mouse fails to survive challenge or if no viable parasites are isolated from the brain, spleen and liver, the mouse is considered to be cured.

Besnoitia jellisoni infected mice are challenged with Semliki Forest Virus to study the resistance of protozoan infected mice to viral challenge.

Major Findings: In addition to the three cases of toxoplasmosis transmitted by transfusion of white blood cells and reported last year one additional case was observed. A positive anti-toxoplasma IgM-IFAT of 1:20 was found in one out of 570 sera obtained from Central Blood Service, Baltimore, via The NIH Blood Bank. Sixty-seven percent of the sera examined were negative at a dilution of 1:18 by the IHA. These findings indicate that the risk of transmitting toxoplasmosis from apparently healthy blood donors is slight.

Chlorinated analogues of lincomycin U-24 (7 chloro-N-demethyl-4'-pentyl-lincomycin) and U21-(7 chlorolincomycin) have been shown to be effective chemotherapeutic agents against toxoplasmosis in mice. No viable parasites were found in 13 of 15 experimentally infected mice 6 weeks after treatment with U-24 at a dose level of 50 mgm/Kg/day for 14 days. Ten out of 14 mice given U21 at 200 mgm/Kg/day were also free of viable toxoplasma when killed. Mice treated with U-24 had negative or low (1:162 or less) IHA titers while those given U21 had titers which ranged from 1:54 to 1:4000. Toxoplasma was isolated only from mice with titers of 1:486 or greater.

Mice infected with B. jellisoni have been shown to be resistant to a lethal challenge of Semliki Forest Virus. Although the resistance is thought to be due to activated macrophages, laboratory findings so far have not been conclusive.

The E. histolytica skin test antigen is currently being evaluated in normal subjects and patients at the Clinical Center (NIAID #69I-127) and in India as part of PL-480 project #854.366.

Significance to Bio-medical Research and the Program of the Institute:

Serious toxoplasmosis occurs occasionally in patients with leukemia. Since their immune responses as well as those of patients receiving organ transplants are often altered, continued studies of toxoplasmosis in such patients should lead to a better understanding of the factors which reactivate dormant chronic infections. Although the risk of transmitting toxoplasmosis via normal blood donors is slight, the risk of transmission via white cell transfusions from donors with chronic myelogenous leukemic (CML) having elevated antitoxoplasma titers is evidently greater. Occurence of parasitemia in this group of patients may be greater because of the combined effects of the disease and immunosuppressive therapy.

The combined use of pyrimethamine and sulfadiazine in the treatment of toxoplasmosis has had some serious hematological side effects such as leukopenia and thrombocytopenia. Pyrimethamine may also be teratogenic. These factors indicate the need for a new drug or combination of drugs with anti-toxoplasma activity. The results obtained with the lincomycin analogues suggest that these compounds may be useful in the treatment of toxoplasmosis.

Continued studies involving concomitant viral infections and Besnoitia or Toxoplasma may lead to a better understanding of the defense mechanisms involved in intracellular parasitism.

Proposed Course of the Project: Through the cooperation of the Leukepheresis Unit and Medicine Branch NCI, and The NIH Blood Bank, the risk of transmitting toxoplasmosis via blood and blood products will be further investigated. Sufficient sera from CML patients has not been obtainable at NIH for determining the risk of leucocyte transmission of toxoplasmosis. Attempts to locate sources of additional CML patients to follow will be made outside NIH.

Chemotherapeutic studies on the effectiveness of chlorinated lincomycin analogues against toxoplasma will be continued to see if these drugs are also effective against the cyst stage.

Cellular immunity will be studied by means of macrophage or leucocyte migration inhbition. The 51Cr (as sodium chromate) will also be used to measure the lytic activity of "sensitized" lymphocytes for the *in vitro* detection of cell mediated immunity.

Additional sera from PL-480; project 854.366 in India will be examined for anti-*E. histolytica* antibodies.

Honors and Awards:

Lectured on the Serological Diagnosis of Toxoplasmosis at the NIH graduate school, Biology 200, Advanced Topics in Microbiology.

Publications:

Lunde, M. N. and Melton, M. L.: Use of the hemagglutination test in the detection of anti-toxoplasma antibodies in mouse serum. *J. Parasit.* 56: 617-618, 1970.

Siegel, S. E., Lunde, M. N., Gelderman, A. H., Halterman, R. H., Brown, J. A., Levine, A. S., and Graw, R. G., Jr.: Trasmission of Toxoplasmosis by Leukocyte Transfusion. *Blood.* 37: 388-394, 1971.

Lunde, M. N. and Gelderman, A. H.: Resistance to Lymphoid Leukemia of AKR Mice Associated with a Chronic Protozoal Infection, *Besnoitia jellisoni*. *J. Natl. Cancer Inst.* (In Press).

Serial No. NIAID 125-Q
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Indvidual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunological aspects of some protozoal infections in laboratory animals

Previous Serial Number: Same

Principal Investigator: Robert Herman

Other Investigators: Tsugiye Shiroishi

Cooperating Units: Laboratory of Viral Diseases (NIAID)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To elucidate the nature of the protective mechanism induced by certain viruses against the exo-erythrocytic form of Plasmodium gallinaceum (avian malaria) (Herman, Baron, Shiroishi and Buckler).

To explain the protective mechanisms induced by synthetic polyribonucleotides (Poly I · Poly C and Poly A · Poly U) in mice infected with the trypanosome, Trypanosoma congolense.

Methods Employed: 1) The effects of several viruses (e.g., Chikungunya, influenza (PR8), and Newcastle disease virus (NDV) upon the exo-erythrocytic form of avian malaria (Plasmodium gallinaceum) is being studied by inoculating these viruses into the allantoic cavities of chick embryos prior to infection with the parasite. Malarial infection of the embryos is assessed by cell culture from infected organs and by inoculation of known amounts of infected embryo liver tissue into young chicks. In addition, the direct effects of chick interferon upon the exo-erythrocytic stage of the parasite are also being studied in a similar manner.

2) Poly I · Poly C, Poly A · Poly U and NDV, a patent inducer, of interferon, were tested for their protective effects against T. congolense in mice by determining parasitemias and mortality of mice infected with various numbers of parasites. The immunosuppressant cyclophosphamide was used to determine whether the protection observed was interferon-mediated or immunologically induced.

Major Findings: Both Chikungunya and influenza virus apparently inhibit the reproduction of the exo-erythrocytic stages of avian malaria in the chick embryo. Whether interferon is directly responsible for the observed protection is being investigated. Neither organs from treated embryos nor their allantoic fluids contain appreciable amounts of interferon or virus. On the other hand, NDV, which grows well in chick embryos, apparently offers no protection against the exo-erythrocytic form of avian malaria in ovo. However, NDV, when inoculated into young chicks confers protection against malaria. The observed viral protection could not be duplicated by exogenous chick interferon (of relatively low titer). Thus protection of the chick embryo against the exo-erythrocytic forms of P. gallinaceum by Chikungunya and influenza (PR-8) viruses may be due to either low levels of interferon, viral interference or a combination of both.

Poly I · Poly C-resistant and Poly I · Poly C-susceptible variants of Trypanosoma congolense ("R" and "S" respectively) were isolated and maintained in mice. Mice infected with the "R" variant showed no response to either polyribonucleotide but those infected with the "S" variant consistently exhibited a decrease in mean parasitemias and cumulative mortality when treated with Poly I · Poly C, but not with Poly A · Poly U. The protective effect of Poly I · Poly C was dose dependent and best when first given around the time of infection of the mice. Thus, using a selected strain of T. congolense, Poly I · Poly C and, under certain circumstances, Poly A · Poly U, but not NDV, could protect mice against infection. This protection was immunologically mediated.

Injections of NDV offered no protection against T. congolense in mice. The protective effects of Poly I · Poly C were reversed by treatment with the immunosuppressant, cyclophosphamide. However, the latter could not reverse such effects in mice infected with Semliki forest virus. This strongly suggests that the protective mechanisms stimulated by Poly I · Poly C in these two infections were different.

Significance to Bio-medical Research and the Program of the Institute:

Although endogenous and exogenous interferon is known to offer some degree of protection against rodent malaria, the mechanisms of such protection are unknown. Use of malaria-infected chick embryos in which the exo-erythrocytic stages of avian malaria are readily observed offers several advantages when compared to malaria in mammals. Most immunological phenomena inherent in mammalian systems are absent from the embryo. Sporozoites of avian malaria are readily available in large numbers. The nature of the viral protection against this species of malaria *in ovo*, offers a new approach to unexplained and unexplored phenomena in the immunology of malaria.

The immunological enhancing capacity of Poly I · Poly C as a protective mechanism against certain strains of the trypanosome, *T. congolense*, has not been studied.

It is not known what effects immunological enhancement induced against one species of trypanosome will have against selected strains or species closely related to those infecting man and his domestic animals. The fact that only certain antigenic strains of *T. congolense* are responsive to the immunological enhancing capacities of Poly I · Poly C is important in that it provides a new tool for studying antigenic variability upon which the parasite's pathogenicity and infectivity depends.

Proposed Course of the Project: 1) Studies are planned to determine the specific nature of the antimalarial capacity of viruses. The effect of non-interferon stimulating viral infections on sporozoite-induced rodent malaria will also be investigated.

2) The effects of malarial infection-induced derangements of the immune processes upon the growth of certain tumors of rodents is being studied.

Honors and Awards:

Invited seminar speaker, Smith, Kline and French, Inc., West Chester, Pa., December 18, 1970. Topic: The effect of interferon and its inducers on experimental protozoan parasitic infection.

Invited speaker, New York Academy of Sciences, Microbiology Section, January 20, 1971. Topic: Interferon and its inducers in protozoan parasitic infection.

Publications:

Jackson, George J., Herman, R. and Singer, I. (Eds.) *Immunity to Parasitic Animals* (Vol. II). Appleton-Century-Crofts, 1970, 1217 pp.

Schmunis, G. and Herman, R.: So-called natural antibodies in various normal sera against culture forms of *Leishmania*. *J. Parasit*. 56: 889-896, 1970.

Schmunis, G. and Herman, R.: Macrophage activity of thymectomized mice infected with *Leishmania donovani*. *Experientia* (In Press).

Serial No. NIAID 131
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Experimental Chemotherapy of Malaria

Previous Serial No.: Same

Principal Investigator: Kendall G. Powers

Other Investigators: Richard L. Jacobs

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	12/12			12/12
Professional	4/12			4/12
Other	8/12			8/12

Project Description:

Objectives: To establish and maintain a series of models for use in evaluating potential antimalarial activity of various compounds. This would include parasites from birds, rodents and non-human primates. Drug activity is evaluated against both blood and tissue stages of the parasite.

Methods Employed: Compounds with antimalarial activity are evaluated orally and intraperitoneally in mice infected with *Plasmodium berghei* and in certain instances in mice infected with *P. vinckei*. Efficacy is based on the percentage of infected erythrocytes in treated mice as compared to untreated controls. When activity warrants, componds are evaluated against blood- and sporozoite-inoculated *P. gallinaceum* infections in chickens and *P. cynomolgi*, *P. knowlesi* and/or *P. coatneyi* infections in rhesus monkeys. A new host parasite system is currently being used to further evaluate compounds. *Aotus trivirgatus* (owl monkey) infected with a chloroquine resistant strain of *P. falciparum* is now available for use in the search for compounds which are effective against chloroquine-resistant falciparum malaria in man.

Compounds may also be tested against strains of P. berghei resistant to chloroquine, pyrimethamine, quinine, DDS or certain long-acting sulfonamides and against P. vinckei resistant to pyrimethamine and chloroquine.

Major Findings: One of the chlorinated lincomycin analogues which possesses both prophylactic and therapeutic activity against mammalian and bird malaria was further tested against a strain of P. falciparum highly resistant to chloroquine. Fifty mg/kg/day oral doses of 7-chloro-N-demethyl-4'-pentyllincomycin (U-24) were administered for seven consecutive days to Aotus trivirgatus infected with chloroquine-resistant falciparum malaria. Control groups received the maximum dose of chloroquine (20 mg/kg/day x 7) tolerated by owl monkeys or the medium in which U-24 was suspended. All monkeys were first treated when about 2% of the erythrocytes were infected. Parasitemias, in monkeys treated with U-24, started to decline after the third treatment and no asexual parasites were detected by the end of the treatment period. Since no recrudescences were observed in any of these monkeys during a 60 day observation period, they were judged cured of the infection. Control monkeys treated with chloroquine or the suspending medium experienced fulminating infections and died.

In earlier studies with P. cynomolgi the lincomycin analogues were slow in clearing parasites from the blood and it was suspected that this slowness of activity would be a problem when treating fulminating infections such as occur with P. falciparum and P. knowlesi infections. This apparent slowness of activity did not appear to be a serious limitation in treating P. falciparum in owl monkeys and it certainly was not a problem in an experiment where rhesus monkeys infected with P. knowlesi were first treated when about 10% of the erythrocytes were infected. In each instance infections were rapidly controlled and all animals survived.

Recently, two of the lincomycin analogues (7-chlorolincomycin and 7-chloro-N-demethyl-4'-pentyllincomycin) have been tested against acute Toxoplasma gondii in mice. Cures have been achieved with both analogues and the data indicate the potential usefulness of these compounds in the treatment of toxoplasmosis. (See Project No. NIAID 125-P)

Significance to Bio-medical Research and the Program of the Institute:

Since the appearance of chloroquine-resistant falciparum malaria in man there has been a world-wide effort to discover an effective compound for treating this disease. Emphasis has been placed on finding compounds which differ chemically and in mode of action from commonly used antimalarials. The cruative activity of 7-chloro-N-demethyl-4'-pentyl lincomycin against chloroquine resistant falciparum malaria illustrates the potential usefulness of this compound and is of particular interest since it represents a major structural departure from compounds commonly used in the treatment of malaria.

Proposed Course of the Project: Additional studies will be conducted using the owl monkey-*P. falciparum* test system to evaluate the antimalarial properties of U-24 at lower dose levels. Another analogue of the series (7-chlorolincomycin) will also be evaluated.

Serial No. NIAID 131-B
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Drug Resistance in Experimental Malaria

Previous Serial No.: Same

Principal Investigator: Richard L. Jacobs

Other Investigators: Kendall G. Powers

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	8/12			8/12
Professional	6/12			6/12
Other	2/12			2/12

Project Description:

Objectives: To investigate the mechanisms of acquired resistance to antimalarial drugs by malarial parasites; to characterize different drug-resistant strains by their cross-resistance patterns to various antimalarial drugs, morphology, pathogenicity, and stability of resistance in the absence of selective drug pressure; to develop effective treatments for drug-resistant infections either through the use of existing drugs in combination or the use of new classes of drugs. Immunologic responses of the host to normal and drug-resistant parasites are examined.

Methods Employed: Beginning with strains of Plasmodium berghei and P. vinckei which were maintained in mice by blood transfer, standard dose-suppression tests were conducted to establish sensitivity of the parent strains to quinine, chloroquine, pyrimethamine and primaquine. Parasitemia was determined in blood smears stained by the Giemsa procedure. Percent parasitemia in treated and control groups were compared to determine the suppressive effect of different doses of drug.

The procedure used to select the various drug-resistant strains involved the intravenous inoculation of groups of mice with large numbers of parasites (up to 20 million parasitized erythrocytes per mouse) and treatment with the highest dose of drug that allowed for some development of parasitemia. Transfer to fresh mice was made as rapidly as feasible, usually by day 7. Drug dosage was increased in successive passages as resistance appeared. The parasite strain was considered to be totally resistant when it was able to develop in mice treated with maximum tolerated doses of the drug. Totally resistant strains were either maintained in treated mice or in the deep freeze. Cross resistance patterns and stability in the absence of selective drug pressure was determined for each resistant strain.

The activity of chlorinated lincomycin derivatives was evaluated against the various resistant and normal strains of P. berghei and against both blood-induced and sporozoite-induced P. cynomolgi infections in rhesus monkeys.

Major Findings: The characteristics of the various drug-resistant strains have been described in previous reports. Strains of P. berghei have been selected that are totally resistant to chloroquine, quinine, pyrimethamine, primaquine, and to both chloroquine and pyrimethamine. Strains of P. vinckei have been selected that are resistant to chloroquine and pyrimethamine. Both of these chloroquine-resistant species develop more slowly and are less frequently fatal to mice than the normal parent strains. This increase in survival rate in mice infected with drug-resistant parasites provides a model for the study of acquired immunity in this host-parasite system without resorting to chemical abbreviation of the infection. In spite of the differences between the chloroquine-resistant and parent strains of P. berghei, we have been unable to demonstrate antigenic differences between these two strains.

Our drug-resistant strains of P. berghei have been tested against the chlorinated lincomycin derivatives U-21 and U-24. Each of the resistant strains, as well as the parent strain, was sensitive to U-21 and U-24. These results lend support to the concept that these compounds differ in their mode of action from the commonly used antimalarials. No attempt has yet been made to select for resistance to these lincomycin derivatives. One of these compounds (U-24) which has been shown to be effective against blood stages of P. cynomolgi in rhesus monkeys, failed to produce a radical cure in monkeys infected by sporozoites. When monkeys became patent after sporozoite inoculation, ten daily doses of U-24 (50 mg/kg/day) resulted in clearance of blood forms of the parasite, but none of the monkeys were cured and recrudescences were seen about 30 days after the treatment period in all treated monkeys. These infections continued to recrudesce even after a course of chloroquine treatment followed by a second treatment with U-24 at 100 mg/kg/day. Thus, treatment after patency did not affect exo-erythrocytic forms of this particular parasite.

Significance to Bio-medical Research and the Program of the Institute:

Drug resistance continues to present a major problem in the chemotherapy of malaria in many areas of the world. Strains of *P. falciparum* that are resistant to all of the commonly used synthetic antimalarials, and also to quinine in some instances, continue to be encountered in widely separated areas of the world. It seems probable that other species of malaria that infect man will become more refractory to presently effective drugs. The emergence of drug resistance in malarial parasites has emphasized the need for a better understanding of the basic mechanisms of drug action against the parasite and other aspects of host-drug-parasite relationships. *P. berghei* and *P. vinckei*, both parasites of rodents, are valuable laboratory models for the investigation of the mode of action of various antimalarial compounds and the basic aspects of acquired drug resistance.

The need for effective means for the effective treatment of drug-resistant malaria is evident and a continuing urgent need for chemotherapeutic agents that are effective against these resistant infections is now realized. Possible temporary solutions to the problem include the use of combinations of existing drugs, but the development of new types of drugs would seem to provide a more permanent solution to the problem.

Proposed Course of the Project: Studies on drug resistance will be continued along the general lines outlined above. Differences between normally sensitive and the various drug-resistant strains will be studied in an attempt to gain a better understanding of the mechanisms of acquired drug resistance and the mode of action of drugs.

Serial No. NIAID 131-C
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Immunity and Pathology of Malaria

Previous Serial No.: Same

Principal Investigator: Kendall G. Powers

Other Investigators: John F. Finerty and P. C. C. Garnham (Guest Worker)

Cooperating Units: Laboratory of Microbial Immunity and London School of Hygiene and Tropical Medicine

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	11/12	2/12	4/12	17/12
Professional	3/12	2/12	4/12	9/12
Other	8/12			8/12

Project Description:

Objectives: To study the immune response and pathological consequences of acute and chronic malaria and other parasitic diseases.

Methods Employed: *Plasmodium cynomolgi* in rhesus monkeys is one of the parasite models used because there is considerable basic information on this species and because of its similarity to *P. vivax* in man. The others are *P. berghi* and *P. vinckei* in mice. Various parameters are examined daily during the active phases of infection, including thick and thin blood smears, hematocrits, and sera are collected for immunologic studies. Immunologic studies include qualitative and quantitative changes in immunoglobulins as well as fluorescent antibody titer changes.

Major Findings: The immunologic response in monkeys injected a single time with *P. cynomolgi cynomolgi* sporozoites was studied. Monkeys were allowed to run a normal untreated course of infection or peripheral parasitemias were suppressed by treatment with twice weekly doses of chloroquine starting at the time of exposure and continuing for 50, 100 or 150 days, respectively. Serum antibodies specific for P. cy. cynomolgi were first detected in untreated monkeys 15 days after exposure (titer 1:32) and maximum titers (1:256) were recorded 12 days later. Although antibody titers generally declined after the first wave of parasitemia, occasional increases were observed which appeared related to periodic relapses during a 200 day observation period. Similarily, serum antibody titers (1:32) were first detected 15 days after exposure in monkeys where peripheral parasitemias were suppressed by chloroquine. In contrast to untreated controls, titers did not increase significantly until chloroquine therapy was discontinued. Shortly thereafter monkeys became patent and both parasitemias and serum antibody titers increased rapidly. Once these monkeys became patent, the rise and fall in both parasitemias and serum antibody production in chloroquine-treated monkeys were almost identical to that seen in the previously described untreated monkeys. It appears that serum antibodies produced prior to patency had little or no effect on the acute course of infection. It is possible that the antigenic stimulation by tissue merizoites, which apparently do not multiply during chloroquine therapy, is quite different from that produced by rapidly multiplying stages during the first wave of parasitemias and antibodies which are subsequently produced are quite different in their mode of action. For example, serum antibody produced during chloroquine treatment might be of low avidity and therefore does not bind well to parasite material. Another unexpected finding was the lack of an anamnestic response when large amounts of antigenic material became available during acute infection in monkeys that already had antibody titers specific for P. cynomolgi. Immunoglobulin levels were measured in treated and untreated monkeys. Only IgM production paralleled the rise and fall in serum antibody titers while IgG increased significantly only after the first wave of parasitemia.

Findings on the affects of various strains of Schistosoma mansoni in rhesus monkeys are included in report No. 121-H.

Significance to Bio-medical Research and the Program of the Institute:

Traditionally, malaria studies have involved the acutely infected individual. Information on the immune status of and the pathological consequences in chronic malaria infections is very sparse. Yet this naturally acquired protection may be the most productive clue to an understanding of the immune responses to infection with this parasite. It is now possible, using various species of malaria parasites in monkeys, to duplicate by repeated exposure of the host the pathologic and immunologic responses which are characteristic of human populations in endemic areas. These studies are basic to our understanding of the ecology and distribution of malaria as well as the action of drugs.

Proposed Course of the Project: Studies involving sporozoites-and blood-induced infections in rhesus monkeys are being continued. The immune response of the host to exo-erythrocytic infections will be evaluated and studies will be continued on the role host immunity plays in malaria relapse.

Honors and Awards:

Invited discussant, section on chemotherapy of protozoan diseases. Second International Congress of Parasitology, Washington, D.C., Sept. 6-12, 1970.

Member of task force on parasitological topics of interest to the Agency for International Development. Washington, D.C., 1970-71.

Secretary-Treasurer: Tropical Medicine Association of Washington,D.C.

Chairman: NIAID Safety Committee, NIH, Bethesda, Maryland.

Executive Committee member: Helminthological Society of Washington.

Publications:

Barker, L. R. and Powers, K. G.: Antilymphocyte serum in rodent malaria: Impairment of antibody response and recovery. Nature 229: 429-430, 1971.

Barker, L. R. and Powers, K. G.: Immunosuppression in rodent malaria: Effect upon recovery and antibody response. Am. J. Trop. Med. & Hyg. 20: 389-393, 1971.

See Serial No. NIAID 121-H for publications on schistosomiasis.

Serial No. NIAID 131-E
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Indvidual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of Host Nurtition on the Course of Experimental Malaria Infection

Previous Serial Number: Same

Principal Investigator: Richard L. Jacobs

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	2/12			2/12
Professional	1/12			1/12
Other	1/12			1/12

Project Description:

Objectives: To investigate the effect of altered host nutrition (protein deficiency) on the course of experimental malaria infections.

Methods Employed: Altered nutritional status was produced in mice by feeding groups of mice purified diets of known composition. Protein deficiency was accomplished by varying the level of casein in the test diets, with the control diet containing 27% casein as compared to 8% in the protein deficient diet. Other essential ingredients did not differ in the two diets. Young (10 to 14 days old) female mice of the NIH General Purpose strain were maintained on the test diets for varying periods of time before infecting with blood forms of Plasmodium berghei (17-X strain) which normally produces non-fatal infections in mice. Prior to infection, the relative degree of protein deficiency was assessed by differences in rate of growth and by typical gross symptoms of protein deficiency. Infection was accomplished by intravenous injection of approximately 5×10^6 parasitized erythrocytes per mouse. Blood films were made daily to evaluate the course of parasitemia. All blood films were stained with Giemsa stain and examined microscopically. Parasitemia is expressed as the percentage of erythrocytes parasitized.

Major Findings: Protein-deficient mice infected with *P. berghei* have been shown to develop more severe infections than mice fed a high-protein diet. In repeated trials the protein-deficient mice have developed slightly higher mean peak parasitemias and more extensive course of parasitemia than that seen in controls. Mortality rate in protein-deficient mice due to the infection is consistently higher than in the control mice. The degree of protein deficiency appears to be of critical importance in altering the severity of infection. Thus, mice started on low protein diets at a very early age (10 days of age) show more severe infections than when placed on test diets when older. As mice become older when maintained on the test diets, less effect of protein malnutrition reflected alteration of the course of infection.

Significance to Bio-medical Research and the Program of the Institute:

Malnutrition in almost any form is generally considered to increase susceptibility to infectious diseases. However, exceptions are known to occur where dietary factors are required by the infecting organism to a greater degree than by the host. Nutritional deficiencies ranging from starvation through kwashiorkor to more specific caloric, vitamin, or mineral deficiencies are widespread in areas of the world where malaria is endemic. However, the specific effects of the various nutritional problems on the severity and the development of immunity to malaria is not well established.

Proposed Course of the Project: This study is terminated.

Serial No. NIAID 131-H
1. Parasitic Diseases
2. Chemotherapy Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Surveys for protozoan parasites of the blood in imported animals.

Previous Serial Number: Same

Principal Investigator: Dr. Kendall G. Powers

Other Investigator: Tsugiye Shiroishi

Cooperating Units: National Zoological Park

Man Years

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	1/12			1/12
Professional	1/12			1/12
Other				

Project Description:

Objectives: To obtain information on the distribution and incidence of blood protozoan infections in as many as possible of the large variety of vertebrates imported into this country. This would include animals brought in for research as well as additions to collections in zoos.

Methods Employed: Blood smears are stained with Giemsa stain and examined for blood protozoans. Most materials are obtained through consultative work with the National Zoological Park, Washington, D. C.

Major Findings: Eighty-six blood films from snakes, birds and mammals were examined for the presence of parasites. All of these samples were from exotic and native species maintained at the National Zoological Park. The following parasites were found:

Source	Parasite
Penguin	Plasmodium relictum
White cheeked Gibbon (Hylobates concolor)	Hepatocystis
Caiman	Microfalaria
Indigo snake	Hemogregarine
Owl Monkey (*Aotus trivirgatus*)	Microfalaria

Significance to Biomedical Research and the Program of the Institute: Malarias from animals other than man have taken on considerable importance from two points of view: (1) Some of the non-human primate forms are infective to man and (2) the need for adequate laboratory models for the study of malaria is sufficient to promote a search for new and different parasites in various parts of the world. The information obtained on various hosts and the geographic distribution of malaria parasites is valuable as a baseline for field studies in areas where human and other primate malarias are endemic.

Proposed Course of the Project: It is recommended that this project be discontinued and that future requests from the National Zoological Park for identification of blood parasites be handled on an informal basis.

Serial No. NIAID 131-I
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Relapse Mechanism in Experimental Malaria

Previous Serial No.: Same

Principal Investigator: Kendall G. Powers

Other Investigators: McWilson Warren and P. C. C. Garnham (Guest Worker)

Cooperating Units: London School of Hygiene and Tropical Medicine

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	8/12		2/12	10/12
Professional	3/12		2/12	5/12
Other	5/12			5/12

Project Description:

Objectives: To investigate the biological and immunological aspects of the relapse phenomenon in malaria. Efforts will be made to clarify the relationships between latency and relapse in malaria.

Methods Employed: The relapse potential of three strains of *P. cynomolgi* (mulligan, ceylonensis or langur) is being studied in the rhesus monkey. 1) Sporozoite-induced infections employ sporozoites from *Anopheles atroparvus* fed on infected monkeys. Infections are monitored by daily thick blood films and monkeys are treated with a single 10 or 20 mg/kg dose of chloroquine base during each relapse in an attempt to lessent the amount of acquired immunity which may develop. Sera are collected weekly and various immunologic evaluations are conducted to detect changes which might be related to relapse. 2) Sporozoites in living mosquitoes are exposed to X-radiation and subsequently injected intravenously into monkeys. These animals are followed as described above. 3) A system for evaluating chemicals against exo-erythrocytic stages of *P. cynomolgi* in rhesus monkeys is also being used. Sporozoites are injected directly into a marked area of the liver and biopsies of these areas are performed at selected days after injection. Animals are treated before or after sporozoite injection depending on the type of information desired.

Major Findings: The long-term relapse study was terminated about 1,000 days after a single sporozoite exposure. The ceylonensis strain lost relapsed on day 1,020, the mulligan strain on day 900 and the langur strain on day 864. These data illustrate the prolonged relapse potential of these vivax-like malaria parasites. The frequency and longevity of relapse appears related to the number of sporozoites injected. Monkeys which received 300,000 sporozoites experienced a much greater number of relapses (22) than any of those infected with 700 (average 9.6) during a 360 day observation period. Monkeys which received the smaller number of sporozoites did not relapse after day 200; apparently they had reached the end of their relapse potential. The data suggest that the number of relapses can be influenced by the number of sporozoites involved in the original inoculum. The fact that sporozoite numbers can directly influence the number of relapses in experimental malaria infections is very difficult to explain in terms of the cyclic maintenance of liver development as proposed in 1948 by Shortt and Garnham since according to this theory one sporozoite could conceivably initiate the entire sequence in a malaria infection and the infection could continue indefinitely. We believe that our data supports a concept of latency as the most probable explanation for such phenomena in malaria as delayed patency and relapse.

During our study of the relapse potential of P. cynomolgi in rhesus monkeys the question arose as to the curative activity of a single 10 or 20 mg/kg dose of chloroquine base given each time parasites appeared in the blood. In order to be sure that subsequent parasitemias originated only from exo-erythrocytic tissues it was essential to determine the dose of chloroquine which eliminates all parasites from the blood. Infected erythrocytes from monkeys infected with the mulligan or langur strain were injected into susceptible monkeys and infections were treated as they became patent. Cures were judged after treated animals had been negative for 60 days; splenectomies were performed on day 30. A single 10 mg/kg dose of chloroquine cured all 6 monkeys infected with the langur strain. A similar 10 mg/kg dose cured 4 out of 6 monkeys infected with the mulligan strain. All six monkeys infected with the latter strain were cured when the dose was increased to 20 mg/kg.

A study was conducted to determine if 7-chloro-N-demethyl-4'-pentyl lincomycin (U-24) had an effect on exo-erythrocytic (EE) stages of development. Monkey livers were injected with viable sporozoites and 5 days later the first of 5 daily 100 mg/kg subcutaneous doses of U-24 were given. Liver biopsies were performed at the site of injection in untreated control and treated monkeys 7 and 10 days after exposure. Although normal appearing EE bodies were present in biopsy material from the untreated monkey no bodies were seen in any of the sections from the animal treated with U-24. Parasitemia first appeared 9 days after sporozoite injection in the untreated animal while no parasites were found in the treated monkey during a 60 day observation period.

It has been reported by Hawkin *et al.* that gametocytes are produced so that they reach a short-lived peak of infectivity at about midnight when mosquitoes are most likely to suck blood. Our experience indicates that there is no such precise pattern of gametocyte infectivity and a carefully controlled study to examine this premise was initiated. A single rhesus monkey was exposed intravenously with erythrocytes infected with *Plasmodium cy. cynomolgi*. After gametocytes appeared in the blood, respective groups of age-matched *Anopheles atroparvus* (50 females/group) were allowed to feed for 30 minutes on the monkey at noon and midnight for 5 days. Mosquito guts were examined and oocysts counted 7 days after each group fed. Infectivity was based on the number of oocysts per mosquito and the percentage of mosquitoes infected. There was no significant difference in gametocyte infectivity when groups of mosquitoes were fed either at noon or midnight. Maximum infectivity of gametocytes occurred 7 and 8 days after exposure which was just prior to peak parasitema of the acute phase of the infection. Although there were more gametocytes in the blood shortly after the peak, the infectivity rate of these gametocytes was much lower than those produced prior to peak parasitemia.

Monkeys in an irradiation experiment were inoculated with about 3 million sporozoites which had received 6.5 to 7.5 Krad. The control was given a similar number of non-irradiated sporozoites. The duration of the study was 500 days. The development of the EE stage can be altered by exposing the sporozoite to x-ray. Changes ranged from apparent retardation to death and the severety of the effect appeared to be dose oriented. Monkeys which received irradiated sporozoites relapsed much less frequently than the control. It was suspected that the frequency of relapse was related more to the numbers of viable sporozoites inoculated than to an alteration of the sporozoite by irradiation.

Significance to Bio-medical Research and the Program of the Institute:

The problems of relapse in *P. vivax* malaria remain among the most serious aspects of international programs against the disease. Drugs which can cure *P. falciparum* bring only temporary parasite clearance and clinical improvement in a patient with *P. vivax*, with eventual clinical and parasitological relapse. Mass prophylaxis programs can be very effective in areas where drug-sensitive *P. falciparum* is found but if *P. vivax* is present, the entire approach must be changed. Relapsing malaria becomes the seed of new outbreaks and causes a long extension of expensive, multi-disciplined antimalarial programs. The nature of the problem is clear, but at the moment the mechanism of relapse in malaria is poorly understood. An understanding of the biology of this phenomenon will broaden the scientific base upon which the concept of malaria eradication is structured, increase the potential for finding curative drugs and may eventually lessen the threat of imported malaria into "malaria'free" areas.

Proposed Course of the Project: A hypothesis of latent stages of the parasite in deep tissues which are responsible for the relapse will continue to be explored. Studies will continue on the relationship of relapse to antigenic variants within the same host and the immune response of the host to malarial infections. These studies will be reported in NIAID 131-C as this project is terminated.

Publications:

Warren, McW.: Simian and Anthropoid Malarias - Their Role in Human Disease. Lab. An. Care 20: 368-376, 1970.

Warren, McW. and Garnham, P. C. C.: *Plasmodium cynomolgi*: X-irradiation and Development of Exo-erythrocytic Schizonts in *Macaca mulatta*. Exper. Parasit. 28: 551-556, 1970.

Serial No. NIAID 131-J
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cyclical Transmission of *Plasmodium berghei*

Previous Serial Number: Same

Principal Investigator: Richard L. Jacobs

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	7/12			7/12
Professional	2/12			2/12
Other	5/12			5/12

Project Description:

Objectives: To develop reliable procedures for the cyclical transmission of various strains of *P. berghei* in mosquitoes and in mammalian hosts. To study the biology of *P. berghei* during cyclical transmission, including the possible relapse potential of this species and the stability of drug resistance during passage through the mosquito.

Methods Employed: Different strains of *P. berghei* which produce viable gametocytes are maintained in mice or in golden hamsters, both by blood inoculation and by sporozoite inoculation. Frozen parasitized blood is stored at -70°C. *Anopheles stephensi* and sometimes *A. quadrimaculus* are employed as mosquito vectors. Experimental hosts include various strains of mice and golden hamsters. Mosquitoes are allowed to feed on the vertebrate host at various stages of the infection to determine the optimal time of infectivity to mosquitoes. After infective blood meal, mosquitoes are maintained at various temperatures to determine the optimal temperature for sporozoite development. Mosquitoes are dissected at various times after the infective blood meal to follow the vevelopment of oocysts on the stomach wall and sporozoites in the salivary glands.

The possible relapse potential of P. berghei was studied in sporozoite-infected mice by suppressing the development of blood forms for various periods of time with either chloroquine or quinine which are active against blood forms of the parasite but do not effect the exo-erythrocytic forms (liver) of the parasite.

The stability of resistance to chloroquine was investigated both during repeated blood passages and sporozoite-induced infections of the 17-X strain.

Major Findings: Reliable procedures have been established for the routine cyclical transmission of both the 17-X strain and NK-65 strains of P. berghei through the mouse, hamster, and Anopheles stephensi. Previous difficulties involving the NK-65 strain seem to have been overcome by using the A/HEN strain of mice. This system provides a parasite strain that is normally sensitive to both quinine and chloroquine that can be routinely passaged through the mouse and mosquito.

As previously seen with the 17-X strain of P. berghei, sporozoite-induced infections with the NK-65 strain fail to demonstrate a clear-cut relapse potential. By appropriate suppressive drug treatment after sporozoite inoculation, the appearance of parasites in the blood can be delayed for periods up to ten days after parasites appear in blood of untreated controls. Whether this delay is due to late developing exo-erythrocytic forms ("relapse") or to the persistence of very low levels of blood forms during drug treatment is not clear, but suggests that this species may possess a relapse potential.

Sporozoite transmission at frequent intervals is necessary in order to maintain gametocyte production in both the 17-X and NK-65 strains. After several successive blood passages in mice these strains fail to produce viable gametocytes and therefore become uninfective to mosquitoes. Also, the 17-X strain, which is naturally resistant to chloroquine, gradually loses this resistance during repeated blood-induced passages in mice. Mosquito passages at frequent intervals are necessary to maintain the 17-X strain in the nonvirulent state for mice.

Significance to Biomedical Research and the Program of the Institute:

A reliable procedure for the routine cyclical transmission of P. berghei through the vertebrate and invertebrate hosts provides a valuable laboratory model for studies on the biology, chemotherapy and immunology of malaria. Establishment of the presence or absence of a relapse potential in this rodent host-parasite model will contribute materially to a better understanding of the biology of this parasite and to interpretation of the effects of various chemotherapeutic agents on the infection.

Proposed Course of the Project: Serial transmission of *P. berghei* from rodent to arthropod vector will be continued so that all stages of the life cycle will be available for study. However, the status of this activity will be terminated as a research project.

Serial No. NIAID 131-N
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Chloroquine Action in Experimental Malaria

Previous Serial Number: Same

Principal Investigator: Richard L. Jacobs

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	7/12			7/12
Professional	3/12			3/12
Other	4/12			4/12

Project Description:

Objectives: To determine plasma levels of chloroquine in rhesus monkeys following different doses of the drug, persistence of chloroquine in plasma after dosing, effect of repeated doses, and plasma levels of chloroquine required to protect against challenge with blood forms of Plasmodium cynomolgi.

Methods Employed: Healthy rhesus monkeys weighing from 4 to 6 kg were treated with single intramuscular doses of chloroquine, 10 or 40 mg/kg. After various time intervals, 10 ml heparinized blood samples were drawn from individual monkeys, 5 ml plasma removed after centrifugation, and cellular components returned intravenously to donor monkeys after dilution with saline. Peak plasma levels of chloroquine were found to occur about four hours after intramuscular administration. Blood was taken daily for seven days after dosing and then every second day until chloroquine became undetectable in the plasma in order to determine persistence after a single dose. In another series of treated monkeys plasma chloroquine levels were again followed and each monkey was inoculated seven days after dosing, intravenously, with blood parasitized with _Plasmodium cynomolgi_. Parasitemia was followed by daily thick and thin blood smears stained by the Giemsa procedure. When parasites appeared in the peripheral blood monkeys were treated with chloroquine, 10 mg/kg, to determine the curative effect of this dose and plasma chloroquine levels were again followed.

Major Findings: Monkeys treated with chloroquine, 10 mg/kg, show peak plasma chloroquine levels of 250 to 500 µg/liter about four hours after the drug is administered. A gradual decline is then seen over a period of about 16 days after which time chloroquine is no longer detectable in the plasma. Intramuscular doses of 40 mg/kg result in peak plasma levels of from 1000 to 3000 µg/liter followed by a sharp drop to very low levels over a period of 16 days. While mean plasma chloroquine levels are related to the dose given, groups of monkeys treated with the same dose show a wide range of differences in plasma levels attained. In monkeys with blood-induced _P. cynomolgi_ infections, single doses of chloroquine at 10 mg/kg was not uniformly curative; 20 mg/kg cured all monkeys of the infection. When given 7 days prior to challenge with blood forms of _P. cynomolgi_, neither 10 nor 40 mg doses of chloroquine protected monkeys against the infection.

Significance to Bio-medical Research and the Program of the Institute:

The rhesus monkey is the most widely used non-human primate in the study of experimental malaria. In this host, _P. cynomolgi_ produces a relapsing type infection which is comparable in many respects to _P. vivax_ in man. In spite of the widespread use of the rhesus monkey infected with _P. cynomolgi_ or other species of malaria parasites in studies on the chemotherapy of malaria, very little information is available regarding the plasma levels of chloroquine required to protect monkeys against malarial infections or on the rate of disappearance of chloroquine from the blood of monkeys. This type of information will be of value for proper evaluation of the effects of chloroquine in this host-parasite system.

Proposed Course of the Project: Project completed.

Serial No. NIAID 131-0
1. Parasitic Diseases
2. Chemotherapy
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathogenesis of disease (Chagas' disease) resulting from infection with *Trypanosoma cruzi*

Previous Serial Number: Same
Principal Investigator: Franklin A. Neva

Other Investigators: Dr. Gustavo Parajon of Hospital Baustista in Managua, Nicaragua

Cooperating Units: Middle America Research Unit, Canal Zone, Panama; Ministry of Health of Nicaragua; and Mr. Earl Fife, Department of Serology of Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	28/12			28/12
Professional	3/12			3/12
Other	25/12			25/12

Project Description:

Objectives: To elucidate some of the factors that determine why infection with *T. cruzi* is associated in some individuals with either acute or chronic disease. To develop improved serologic procedures for diagnosis of infection and/or disease due to *T. cruzi*.

Methods Employed: A number of strains of trypanosomes are maintained cultures, others by syringe passage, and some are maintained in serial passage alternating between mice and vector bugs. The biologic characteristics of strains kept in mouse-bug passage are also tested at intervals by checking peripheral parasitemia and histopathology of mice. Colonies of two species of triatomid bugs are maintained. One strain of *T. cruzi* is passaged in cell cultures of embryonic beef tissue, and tissue phases of the parasite (mainly amastigotes and trypomastigotes) are harvested for antigen. Complement-fixation and hemagglutination tests are done in a standard manner. Blood specimens and triatomid bugs were collected in a field survey of several rural areas of Nicaragua conducted with the cooperation and assistance of the Nicaraguan Ministry of Health, a physician at the Baptist Hospital in Managua (Dr. Parajon) and a MARU Public Health nurse (V. Rivera). During the survey, portable chest X-rays, electrocardiograms and examinations of the heart were performed on selected individuals.

Major Findings:

Field study in Nicaragua: While the principal investigator was in Panama, a field study was planned and carried out to determine the prevalence of infection with *T. cruzi* in selected areas of Nicaragua and relate this to prevalence of disease caused by the parasite (Chagas' disease) as manifested by heart disease. A house-to-house study in 4 different rural areas of Nicaragua yielded 550 serum specimens; finding of vector bugs in 0 to 33 percent of 142 houses examined; portable chest films on 270 individuals >15 years of age; and cardiac examinations and electrocardiograms on 100 adults. The serum samples have all been tested for CF antibodies to *T. cruzi* and show a high prevalence of reactors. Eight isolates of *T. rangeli* were made from 215 suitable blood clots that were cultured. The presence of *T. rangeli* in some of the bugs collected and *T. cruzi* in others has been found. The clinical evaluation of patients for known or suspected heart disease and correlation of these findings with CF tests for Chagas' disease is still in progress.

Antigen from *T. cruzi* from infected cell cultures: The >500 Nicaraguan survey serum samples were tested with multiple *T. cruzi* CF antigens, including a standard purified protein antigen (Fife and Kent), conventional crithidial antigens, and the tissue culture antigen. There was a very close correlation in positive reactions to the purified protein antigen and the tissue culture antigen, but serum titers were several dilutions higher with the latter antigen. Considerably more reactors, but at low dilutions, were detected with the conventional crithidial antigen, suggesting non-specific reactions. The degree of cross-reactivity exhibited by the tissue culture CF antigen with sera from leishmaniasis cases is not yet settled; at least one batch of tissue culture antigen appeared to be cross-reactive, but other batches have not cross-reacted.

Comparative behavior of different strains of *T. cruzi*: Much of the data from several years previous work has now been organized, tabulated, and is in various stages of analysis. The data concerning infection of cell cultures by newly isolated strains is ready to be assembled for publication, but associated histopathologic findings with these strains still await analysis.

Significance to Biomedical Research and the Program of the Institute:

The optimum antigen for serodiagnosis of *T. cruzi* infection is still an unsettled matter, so improvements in this area would be of great practical value. Serologic tests for Chagas' disease are an important and frequent clinical laboratory procedure in much of Latin America. There is also considerable basic scientific interest in the possibility that forms of the parasite in cell cultures are antigenically different from flagellate culture forms from which current antigens are prepared. One reason for carrying out the Chagas' disease study in Nicaragua was to obtain a large number of serum samples for evaluating our tissue culture T. cruzi antigen. The Nicaragua survey will also provide the best information available to this time concerning frequency of Chagas' disease in that country. In addition, the study was designed to correlate serologic evidence of T. cruzi infection with information on prevalence of disease due to the parasite. This approach, which attempts to assess the impact of a parasitic disease upon the health of a community, has been virtually neglected in dealing with Chagas' disease. More definitive studies of the host-parasite interaction at the cellular level, using a tissue culture adapted strain of T. cruzi and some of the general approaches employed earlier, have been undertaken by Drs. Dvorak and Hyde.

Proposed Course of the Project: The tissue culture T. cruzi antigen still has not been compared to conventional antigens by other immunologic methods. The clinical evaluation of patients with known and suspected heart disease encountered in the Nicaragua study is not yet completed. The study of differences in strains will be assembled for publication and some aspects of it will continue.

Honors and Awards:

Participant in a meeting of a Study Group organized by the Pan American Health Organization on Chagas' disease in San Jose, Costa Rica, December 8-18, 1970.

Serial No. NIAID 133
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Susceptibility of Anopheline Mosquitoes to Simian and Human Malaria Infections and Their Ability to Transmit to Simian and Human Hosts.

Previous Serial Number: Same

Principal Investigators: William E. Collins and Peter G. Contacos

Other Investigators: Wojciech A. Krotoski and William A. Howard

Cooperating Units: Unit on Human Malaria, Laboratory of Parasitic Diseases, Atlanta, Georgia.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	65/12			65/12
Professional	6/12			6/12
Other	59/12			59/12

Project Description:

Objectives: (1) To determine the relative susceptibility of anopheline mosquitoes to simian and human malaria infection; (2) to determine the rate and degree of parasite development within the various mosquito hosts; (3) to attempt transmission of the various simian malarias to a wide spectrum of simian hosts by the bites of infected mosquitoes; and (4) to provide mosquitoes infected with simian malaria for transmission studies in prison inmate volunteers.

Methods Employed: Isolates of simian malaria are maintained either in monkey hosts or in the frozen state for use in transmission studies. Malaria species under study include *Plasmodium brasilianum* (2 strains), *P. cynomolgi* (9 strains), *P. coatneyi*, *P. fieldi* (3 strains), *P. simiovale*, *P. simium*, *P. fragile* (2 strains), *P. gonderi*, *P. knowlesi* (4 strains), *P. inui* (17 strains), *P. jefferyi* and *P. hylobati*. Studies are also made on *P. falciparum*

(5 strains), P. vivax (9 strains) and P. malariae maintained in monkeys. Various species of Anopheles (A. maculatus, A. stephensi, A. freeborni, A. balabacensis balabacensis, A. quadrimaculatus and A. atroparvus) mosquitoes are allowed to feed on infected simian and human hosts. Infection potential to a given mosquito species of malaria in a variety of hosts is determined and transmission by direct mosquito bites are made. Occasionally, when numbers of mosquitoes are low and gland dissections are positive, attempts to infect monkeys are made by the intravenous inoculation of sporozoites from gland dissections. Mosquito susceptibility studies with the human malarias are conducted in cooperation with our Unit on Human Malaria at the U. S. Penitentiary, Atlanta, Georgia.

Major Findings: The results of the transmission studies with various malarias are as follows:

P. knowlesi - A. hackeri strain: Transmission was obtained on 17 occasions with A. b. balabacensis mosquitoes. The prepatent periods ranged from 5 to 8 days with a mean of 6.2 days.

P. knowlesi - H strain: Transmission was obtained on one occasion with A. b. balabacensis mosquitoes. The prepatent period was 5 days.

P. cynomolgi - Bastianellii strain: Transmission was obtained with A. maculatus mosquitoes 15 times and with A. b. balabacensis mosquitoes 5 times. The prepatent periods ranged from 7 to 13 days with a mean of 10.9 days.

P. cynomolgi - Langur strain: Transmission was obtained on two occasions with A. maculatus mosquitoes. The prepatent periods were 11 days,

P. cynomolgi - ceylonensis strain: Transmission was obtained on one occasion with sporozoites from A. stephensi mosquitoes which had been kept frozen for 1,765 days. The prepatent period was 12 days.

P. inui - Mulligan strain: Transmission was obtained on four occasions with A. b. balabacensis mosquitoes. The prepatent periods were 14, 15, 15 and 17 days.

P. inui - OS strain: Transmission was obtained on three occasions with A. b. balabacensis mosquitoes. The prepatent periods were 13, 15 and 19 days.

P. simiovale: Transmission was obtained on six occasions with A. b. balabacensis mosquitoes. The prepatent periods ranged from 11 to 14 days with a mean of 12.2 days.

P. coatneyi: Transmission was obtained once with A. b. balabacensis mosquitoes. The prepatent period was 14 days.

P. vivax - Salvador II strain: Transmission to another monkey was obtained once with A. freeborni mosquitoes. The prepatent period was 42 days.

P. simium: Transmission was obtained on three occasions with A. freeborni mosquitoes. The prepatent periods were 12, 17 and 19 days.

Studies on the mechanisms of relapse indicate that sporozoite induced infections of P. simiovale will relapse from 2 to 6 times in a six month period. This points-up the biological relationship between this species and P. fieldi of monkeys and P. ovale of man.

Newer isolates of P. vivax and P. falciparum continue to infect various species of mosquitoes. Long term feeding studies indicate that infections in the Aotus monkey will infect mosquitoes almost continuously for at least eight months and possibly longer.

Significance to Bio-medical Research and the Program of the Institute:

This project is part of an integrated program of the Laboratory of Parasitic Diseases in the study of simian malaria in monkeys and its relationship to man and human malaria. A clear-cut delineation of the parasite-vector relationship is essential to the proper evaluation of the potential significance of simian malarias as zoonoses in human populations. Detailed laboratory studies on the biology of the simian parasites in the mosquito host are necessary as a supplement to the work being done by other units. Further, there is need for varied species of simian malaria, simulating all of the human species in life pattern and morphology, to carry on significant studies on the chemotherapy, immunology and other aspects of malaria research. A full exposition of the host-parasite-vector relationships of the available monkey parasites is a necessity in providing these useful host-parasite combinations. The availability of infections of human malarias in monkeys makes it possible to study the effect of drugs on the ability of these parasites to infect mosquitoes. It serves also as a source of P. vivax, P. malariae and P. falciparum infected mosquitoes for use in drug studies in man.

Proposed Course of the Project: These studies will be conducted and expanded to include the evaluation of a number of anopheline species of mosquitoes in relation to their potentials as vectors of species of simian and human malaria. Efforts will be directed towards obtaining large numbers of gland positive mosquitoes of several simian species of malaria for use in inoculating monkeys for tissue parasite studies. Efforts will be continued towards developing more effective and efficient means not only of producing larger numbers of mosquitoes for laboratory transmission studies but also of increasing the longevity of such mosquitoes.

Publications:

Contacos, P. G., Coatney, G. R., Orihel, T. C., Collins, W. E., Chin, W., and Jeter, M. H.: Transmission of *Plasmodium schwetzi* from the chimpanzee to man by mosquito bite. *Am. J. Trop. Med. & Hyg.*, 19: 190-195, 1970.

Contacos, P. G.: Primate Malarias: Man and Monkeys. *J. Wildlife Dis.*, 6: 323-328, 1970.

Collins, W. E. and Contacos, P. G.: Observations on the relapse activity of Plasmodium fieldi in the rhesus monkey. *J. Parasit.*, 57: , 1971.

Collins, W. E., Contacos, P. G., Skinner, J. C., and Guinn, E. G.: Studies on the transmission of simian malaria. IV. Further studies on the transmission of *Plasmodium knowlesi* by *Anopheles balabacensis balabacensis*. *J. Parasit.* (In Press)

Collins, W. E., Contacos, P. G., Garnham, P. C. C., Warren, McW., and Skinner, J. C.: *Plasmodium hylobati*: A malaria parasite of the gibbon. *J. Parasit.* (In Press)

Coatney, G. R., Collins, W. E., Warren, McW., and Contacos, P. G.: *The Primate Malarias.* U. S. Department of Health, Education and Welfare, Public Health Service Monograph, 28 Chapters, 1971 (In Press).

Serial No. NIAID 133-A
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Exoerythrocytic Stages of Primate Malaria

Previous Serial Number: Same

Principal Investigator: Peter G. Contacos and William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	14/12			14/12
Professional	2/12			2/12
Other	12/12			12/12

Project Description:

Objectives: (1) To describe the exoerythrocytic (EE) stages of species and strains of malaria in which they have not previously been described; (2) to study the histochemical reaction of EE bodies; (3) to study the prophylactic and curative effects of antimalarial drugs on the EE stages of malaria parasites and to determine the morphologic alterations within the EE stages due to antimalarial drugs.

Methods Employed: Exoerythrocytic forms are produced in a new host by transmission through a mosquito vector from a previously infected host. This is accomplished: (1) by mosquito bite; (2) by direct inoculation of the sporozoites into the liver; or (3) by intravenous inoculation of sporozoites.

Livers are biopsied on selected days after exposure or following drug therapy. Biopsies are processed after paraffin imbedding, as frozen sections or by freeze substitution.

Major Findings: Forty-one liver biopsies were made in three species of non-human primates in attempts to find exoerythrocytic (EE) stages of different species of malaria parasites. Twenty-seven of these were shown to contain EE bodies.

The EE stages of Plasmodium simium, a parasite of South American monkeys, were observed for the first time from Saimiri sciureus and Aotus trivirgatus monkeys. Only seven day forms have been seen to date.

The EE bodies of P. knowlesi were extensively studied to determine their rate of growth in liver tissue. Bodies were found at 12 different 8-hour time intervals ranging from 48 to 136 hours after inoculation of sporozoites into Macaca mulatta (rhesus) monkeys.

The EE bodies of P. simiovale, an ovale like parasite of macaques, were found 7, 8, 9, 10 and 11 days after inoculation of sporozoites into M. mulatta monkeys.

Of major significance was the finding of EE bodies of P. vivax in the A.trivirgatus monkey. Only the seven day forms have thus far been observed.

Significance to Bio-medical Research and the Program of the Institute:

Since the earliest use of antimalarial chemotherapeutic agents, it has been recognized that relapses often occur in previously treated patients. It is now understood that these relapses occur because of the release of forms from the exoerythrocytic stages, these stages having been resistant to the therapy. Therefore, it is extremely important that the activity of any new candidate antimalarial drug against the exoerythrocytic stages be known. Previously, the number of host-parasite models available for the study of this drug activity in primates has been limited. The current project is providing the necessary information and techniques to enable investigators to utilize a number of additional parasite species and primate hosts to evaluate the activity of candidate drugs against pre-erythrocytic stages of the parasite.

Proposed Course of the Project: (1) The demonstration of yet undescribed exoerythrocytic stages of the different primate malarias; (2) the study of the morphologic effects of new antimalarial drugs on the EE bodies.

Publications:

Sodeman, T., Schnitzer, B., Contacos, P., and Durkee, T.: Fine structure of the exoerythrocytic stage of Plasmodium cynomolgi. Science, 170: 340-341, 1970.

Sodeman, T. M., Contacos, P. G., Jumper, J. R., and Smith, C. S.: Studies of the exoerythrocytic stages of simian malaria VI. Plasmodium hylobati. J. Parasit. (In Press)

Coatney, G. R., Collins, W. E., Warren, McW., and Contacos, P. G.: The Primate Malarias. U. S. Department of Health, Education and Welfare, Public Health Service Monograph, 28 Chapters, 1971 (In Press).

Collins, W. E., Contacos, P. G., and Jumper, J. R.: Exoerythrocytic stages of *Plasmodium vivax* in the *Aotus trivirgatus* monkey. *Science* (In Press).

Serial No. NIAID 133-B
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immuno-ecology of Primate Malaria

Previous Serial Number: Same

Principal Investigator: William E. Collins

Other Investigators: None

Cooperating Units: Central America Malaria Research Station, El Salvador

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	12/12			12/12
Professional	2/12			2/12
Other	10/12			10/12

Project Description:

Objectives: To explore the total ecology of malaria and to correlate circulatin antibodies with the presence of parasites and to orient both of these factors with transmission cycles, level of endemicity, and clinical disease.

Methods Employed: Blood samples, on filter paper, are collected from human populations in areas endemic for malaria. These specimens are mailed back to the central laboratory in Chamblee, Georgia for determination of fluorescent antibody titers. The accompanying field studies vary in scope, but wherever possible the following investigations are carried out: (1) Giemsa-stained blood films of the human population for parasite rates and species identification; (2) surveys for malaria parasites in mammals other than man; and (3) studies on the anopheline fauna, including identification, seasonal distribution, biology and malaria infection rate.

Major Findings: In a longitudinal study of inhabitants of the Mato

Grosso area of Brazil, it was found that the IFA technique could give an accurate picture of the malarial experience of the population which was not evident by single malariometric survey. The initial IFA examination of the population indicated that approximately 10 percent of the population had experienced malarial infection and that the predominant species was Plasmodium falciparum. Since the collections were made during a period of very low transmission, the slide examinations indicated only 11 out of 1475 people had malaria and 10 of these were infected with P. vivax. Two subsequent collections have indicated that the IFA results gave a true assessment of the incidence of malaria and distribution of species in the population, thereby emphasizing the usefulness of serologic examinations.

Extensive studies on populations living in different areas of El Salvador have indicated the frequency of species distribution in populations living in zones of differing malarial endemicity. These populations are now being subjected to different malaria control procedures and changes will be measured using IFA techniques.

Significance to Bio-medical Research and the Program of the Institute:

Immunological studies using the FA technique offer an opportunity to determine prior experience with malaria of populations in areas endemic for malaria. The opportunity to correlate field studies with laboratory investigations greatly expands our potential for study on the basic problems associated with human malaria.

This type of investigation gives insight into the mechanisms of the development of protective responses in populations naturally infected with malaria. Information derived from these studies will eventually enable us to predict the future course of malaria in areas of high endemicity and possibly to alert health authorities on the developing potential of epidemics.

Proposed Course of the Project: Cooperative studies will be continued with the Central America Malaria Research Station, El Salvador. These investigations will be expanded to other areas of Africa and South America as our techniques improve and the occasions arise.

Publications:

Collins, W. E., Warren, McW., and Skinner, J. C.: Serological malaria survey in the Ethiopian highlands. Am. J. Trop. Med. & Hyg., 20: 199-205, 1971.

Warren, McW., Cheong, W. H., Fredericks, H. K., and Coatney, G. R.: Cycles of jungle malaria in West Malaysia. Am. J. Trop. Med. & Hyg., 19: 383-393, 1970.

Serial No. NIAID 133-C
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Fluorescent Antibody Studies in Simian and Human Malaria

Previous Serial Number: Same

Principal Investigator: William E. Collins

Other Investigators: Peter G. Contacos and William Butler

Cooperating Units: Unit on Human Malaria, Laboratory of Parasitic Diseases, Atlanta, Georgia; Methodist Hospital, Houston, Texas.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	10/12			10/12
Professional	1/12			1/12
Other	9/12			9/12

Project Description:

Objectives: (1) To determine the development and long-term persistence of specific antibody in malaria-infected humans and monkeys; (2) to study the immunologic relationships between the different species of human and simian malarias; and (3) to determine the effect of biological and surgical immunosuppression on the development and persistence of antibody reactions.

Methods Employed: Serum samples are taken from patients and animals prior to infection and at regular intervals thereafter to determine by fluorescent antibody methods the rate of specific antibody production and the persistence and titer of antibody during the months and years following termination of the malaria infection. Animals having past malarial infection are challenged with heterologous species and strains of Plasmodium sp. and the immunologic response studied by the fluorescent antibody technique. Animals are given curative and sucurative treatments with antimalarial drugs and the effect measured using the FA technique. Animals splenectomized or otherwise surgically treated before and after infection are studied in regard to the FA response. Sera from patients infected with malaria are studied using the specifically labeled immune globulins to determine the patterns of response to each.

Major Findings: Further studies were made on the effect of anti-human thymocyte globulin (HAHTG) on a monkey (*Macaca mulatta*) - malaria (*Plasmodium fieldi*) system. Parasitemias in animals treated with HAHTG were greatly increased over parasitemias in control untreated animals. In contrast, however, monkeys inoculated with HAHTG previously absorbed with monkey red blood cells exhibited parasitemias much lower than did control animals. The reasons for these differences are not, as yet, clear. Fluorescent antibody levels were delayed only slightly by treatment with HAHTG.

As a service to VA Hospitals, State Health Laboratories and the Center for Disease Control, approximately 150 sera from patients with possible malaria infections were evaluated using four different *Plasmodium* antigens (*P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae*).

Significance to Bio-medical Research and the Program of the Institute:

There continues to be a need for additional information on the immune response of the primate host to malaria. The immunological studies using the fluorescent antibody technique offer an opportunity to measure the effect of prior experience with malaria on current clinical attack and to determine the presence of malarial experience in an endemic population. One method in elucidating the immune response is by the observation of the results of selected surgical and biological methods of suppressing immunity. The present study seeks to use these methods in exploration of the mechanisms of immunity to malaria. One of the problems which has been of interest to malariologists is that of separation of species and strains of parasite, including the question of possible identity of species infecting man and lower primates, the utilization of subspecific designations for various strains, and the possible justification of new species from strains or subspecies. The FA studies may provide additional criteria for grouping the different strains, subspecies and species of primate malaria.

Proposed Course of the Project: Further efforts will be directed toward studying the effect of immuno-suppressant drugs and anti-lymphocyte serums on the immunologic response to malaria infection. Studies will continue on the development and persistence of the antibody response in monkeys and man infected with different species of malaria and to determine the effect of multiple infection and chemotherapy on antibody response.

Publications:

Collins, W. E., Contacos, P. G., Skinner, J. C., Harrison, A. J., and Gell, L. S.: Patterns of antibody and serum proteins in experimentally induced human malaria. Trans. Roy. Soc. Trop. Med. & Hyg., 65: 1971.

Collins, W. E., Waren, McW., Skinner, J. C., and Alling, D. W.: Plasmodium inui: Serologic relationships of Asian isolates. Exp. Parasit., 27: 507-515, 1970.

Serial No. NIAID 133-D
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunological Studies on Primate Malaria

Previous Serial Number: Same

Principal Investigators: Peter G. Contacos and Wojciech A. Krotoski

Other Investigator: William E. Collins

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	32/12			32/12
Professional	4/12			4/12
Other	28/12			28/12

Project Description:

Objectives: (1) To evaluate the possibilities for effective biologic prophylaxis against malaria; (2) to analyze the antigenic composition of various species and/or strains of simian malaria; and (3) to localize (insofar as possible) the morphological and/or antigenic unit(s) principally responsible for specific immunofluorescent reactions following naturally acquired malarial infections.

Methods Employed: For the antigenic analysis and the active immunization studies, purified parasite suspensions are obtained from monkeys at the time of peak parasitemia. Packed parasitized erythrocytes are treated with saponin. The parasites are then washed repeatedly with buffered saline. The packed parasites are either stored at -70°C or in the lyophilized state. The parasites are crushed in a Hughes tissue press and the antigenic components extracted by mixing with dilute salt solutions. Another method utilized in the preparation of antigen is to repeatedly freeze and thaw the parasitized erythrocytes. The parasitic and erythrocytic stroma are removed by centrifugation, and the soluble malarial antigens used for various purposes. The antigens prepared are studied by gel filtration chromatography. In addition, parasitized erythrocytes treated with formalin (0.1%) are used as antigen in immunization experiments. Monkeys are actively immunized with protein extracts, with intact parasitized erythrocytes, or with intact parasites freed from the red blood cell by lysis, with fractions of hemolysates from parasitized erythrocytes, or with residue of extracted ruptured parasites. After a suitable period of time, the monkeys are challenged with malaria. Presence of antibody in the serum of the immunized animals is confirmed by indirect FA and immunoelectrophoresis. Antigenic composition of the various preparations is studied by immunoelectrophoresis. Passive immunization is studied by administering immune globulin to monkeys in a dose of 100 mg/kg. The gamma globulin is prepared from blood obtained from monkeys evidencing a degree of immunity to malaria. The ion-exchange batch technique is utilized in preparing relatively pure gamma globulin. The gamma globulin is given prior to exposing the monkeys to bites of heavily infected mosquitoes and at 15 day intervals for 3 doses. Fractionation of hemolysates from parasitized erythrocytes and aqueous extracts of parasites is accomplished by column gel filtration.

Major Findings: Eleven monkeys have been immunized against the M strain of *Plasmodium inui* either with extract, parasites freed from erythrocytes, or parasite residue with from 3 to 6 immunizing doses over periods of time ranging from 2 to 8 months. One monkey has been immunized with serum Soluble antigen (SSA) of *P. knowlesi*. All immunizing doses were in combination with adjuvant (complete Freund's, incomplete Freund's, sodium alginate). Immunized animals in this series have exhibited, after challenge, prepatent periods and courses of parasitemia comparable in all respects to infections in control animals. However, one monkey which received six immunizing doses with Complete Freund's and had ulcerating abscesses at the injection sites did show a low patent parasitemia (maximum of $700/mm^3$ blood).

Of interest is the fact that monkeys receiving immunizing doses of antigen develop strong precipitating arcs (often up to 10) and/or FA titer. However, the presence of these antibodies (precipitating and FA) apparently is not sufficient to exert significant protective effect.

Therefore, there have been no significant changes from our previous observations that immunization of monkeys using a variety of immunizing materials does not completely protect from infection.

Significance to Bio-medical Research and the Program of the Institute:

Until recently the availability of reliable and effective antimalarial chemotherapy obviated the necessity for intensive immunologic studies relating primarily to biologic prophylaxis against amlaria. However, with the advent of some strains of falciparum malaria resistant not only to all the accepted synthetic antimalarials but also to quinine, the need for such studies became obvious. The effectiveness with which passive or active immunization will protect monkeys against infection, not only to the homologous malaria parasite but more especially to the heterologous parasite, should indicate the feasibility of effective biologic prophylaxis against malaria in man.

Proposed Course of the Project: The studies will be continued with further emphasis on separation of antigenic fractions by means of column chromatography. These individual fractions will then be tested for their immunizing ability against malaria. Emphasis will also be placed on serum soluble antigens as immunizing agents as well as immunization by irradiated sporozoites (See Project Report No. NIAID 133-F).

Serial No. NIAID 133-E
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunological Studies on the Sporozoites of Primate Malarias

Previous Serial Number: Same

Principal Investigator: Wojciech A. Krotoski

Other Investigators: William E. Collins and Peter G. Contacos

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	5/12			5/12
Professional	2/12			2/12
Other	3/12			3/12

Project Description:

Objectives: To evaluate the immunological response of normally susceptible primate hosts to immunization with inactivated sporozoites in terms of (1) immunogenic potential, (2) complexity of the response, (3) effect on the parasite, (4) protection from naturally-induced infections, and (5) stage and species specificities.

Methods Employed: Infected mosquitoes are mechanically disrupted in formalinized solutions, and the resultant suspensions subjected to density gradient centrifugation in order to obtain sporozoite-rich fractions. These preparations are employed for inoculation into susceptible monkeys, and for subsequent testing in vitro of host response. Non-formalinized sporozoite preparations are also obtained for comparative testing.

At various intervals after immunization, host susceptibility to infection is evaluated by challenge by sporozoite inoculations. Host humoral responses are evaluated in terms of (1) effect on sporozoites in vitro, (2) fluorescent antibody responses, employing (a) sporozoites and (b) trophozoites as antigens, (3) species specificities, and (4) precipitin responses with trophozoite and/or sporozoite extracts.

Major Findings: Preliminary studies have demonstrated the feasibility of obtaining satisfactory numbers of sporozoites when sufficiently high degrees of mosquito infections have been attained. Sporozoite films of adequate density for FA testing have been prepared, and are now awaiting the completion of monkey immunizations. The greatest single difficulty in this study is that of procuring sufficient numbers of mosquitoes undergoing the high degree of infection desirable for maximum yield of sporozoites. In several experiments, multiple injections of formalinized sporozoites did not significantly alter the prepatent period of mosquito-transmitted *P. cynomolgi* malaria.

Significance to Bio-medical Research and the Program of the Institute:

Mosquito-bite injection of viable sporozoites into susceptible hosts normally results in a rapid metamorphosis of the former into exo-erythrocytic stages and a subsequent patent parasitemia. If specific antibody-stimulating substances are indeed present in the sporozoite, the latter could possess an exploitable immunoprotective potential.

Proposed Course of the Project: Sporozoite purification procedures and immunization of monkeys with the material obtained will be performed on several species of primate plasmodia. Depending on the outcome of these experiments, an eventual extension to studies in man could be anticipated.

Serial No. NIAID 133-F
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of the Efficacy of Immunizing Against Malaria Using Irradiated Sporozoites

Previous Serial Number: None

Principal Investigators: Peter G. Contacos and William E. Collins

Other Investigators: Wojciech A. Krotoski and William A. Howard

Cooperating Units: Unit on Human Malaria, Laboratory of Parasitic Diseases, Atlanta, Georgia; Georgia Tech University

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	5/12			5/12
Professional	2/12			2/12
Other	3/12			3/12

Project Description:

Objectives: (1) To evaluate in monkeys the efficacy of immunization against malaria by inoculation of irradiated sporozoites; (2) to examine the feasibility of immunization of man against homologous and heterologous species of malaria using irradiated sporozoites.

Methods Employed: Mosquitoes are infected with malaria parasites by feeding on infected monkeys. When sporozoites are present, mosquitoes are exposed to varying doses of X-irradiation. Sporozoites are inoculated into test monkeys by the bite of the X-irradiated mosquitoes or by the I.V. inoculation of irradiated sporozoites. After different regimens of immunization, the animals are challenged by the bites of mosquitoes infected with normal malaria parasites. The effect of immunization is measured by changes in prepatent period and course of the parasitemia in addition to serologic techniques. When, and if, a protective effect is shown by one or more of the immunizing procedures, attempts to show a similar protection in human volunteers will be taken under consideration.

Major Findings: Preliminary results in Macaca mulatta monkeys have indicated a prolongation of the prepatent period and a flattening of the parasitemia curve in some animals immunized by multiple inoculations of sporozoites of Plasmodium cynomolgi (B Strain) exposed to 13 K- rads of X-irradiation.

Significance to Bio-medical Research and the Program of the Institute:

With the advent of strains of malaria, particularly P. falciparum, resistant to currently available antimalarial drugs, the urgent need for biologic prophylaxis became obvious. These studies using a monkey-malaria model are an extension of studies by other workers using the rodent-malaria system. If the efficacy and feasibility of such an immunizing procedure can be shown for primate malarias, the effect on the control and/or eradication of malaria would be highly significant.

Proposed Course of the Project: The studies will be continued using the monkey-malaria system. They will be expanded to consider minimum number of immunizing doses required, duration and degree of protection conferred, etc.

Publications:

In manuscript.

Serial No. NIAID 133-G
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Application of Specific Anti-monkey (rhesus) Immunoglobulin Sera to Immunofluorescent and Precipitin Studies in Simian Malarias

Previous Serial Number: None

Principal Investigator: Wojciech A. Krotoski

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	1/12			1/12
Professional	1/12			1/12
Other				

Project Description:

Objectives: To evaluate the immune responses of monkeys to malarial infection by means of anti-rhesus immunoglobulin-sera of sufficient specificity for use in indirect immunofluorescence and precipitin tests.

Methods Employed: Monkey sera (*Macaca mulatta*) are fractionated by gel-filtration on Sephadex G-200 into three main peaks. Peak I contains very high molecular weight proteins, including IgM; Peak II contains middle-range proteins, including IgG and IgA; and Peak III contains mostly albumin, hemoglobin, and similar-sized molecules. Peak I and Peak II proteins are refractionated after concentration on Sephadex G-25, and the contained immunoglobulins are identified by immunoelectrophoretic analysis (IEA) against pooled anti-whole rhesus blood-serum and commercially available specific goat anti-human IgA, IgG and IgM sera. Fractions containing peak amounts of IgM are used for immunization of rabbits, while fractions containing peak amounts of IgG and IgA are partly separated by a stepwise (bulk) elution method from DEAE - Sephadex. Subsequent purification, as required, is accomplished by ion-exchange chromatography. The specific immunoglobulin-enriched fractions are used for rabbit immunization; anti-sera thus obtained are absorbed by serum components present in peaks adjacent to those containing the Ig's, and are further purified by specific immunoadsorption onto cross-reacting human immunoglobulins conjugated to Sepharose. The specific anti-rhesus immunoglobulin sera are then conjugated with fluorescent dyes as needed, or used unconjugated in precipitin tests. Monkeys infected with various species of malaria are bled periodically for determination of serum changes.

Major Findings: This project is in too early a stage to report any significant major findings.

Significance to Bio-medical Research and the Program of the Institute:

Although sufficient antigenic similarities exist between human and rhesus immunoglobulins to permit approximate assay of indirect monkey FA titers with anti-human Ig reagents, a greater sensitivity can be expected with the homologous anti-sera. The preparation of these reagents will permit the assay of monkey Ig's in indirect FA and precipitin testing in simian malarias with a greater degree of accuracy. This, in turn, will lead to a more detailed elucidation of the immune response in the monkey to infection with various species of malaria serving as a counterpart to human studies.

Proposed Course of the Project: The immune response in terms of IgM, IgG and IgA levels will be evaluated by fluorescent antibody and/or precipitating antibody techniques, and the results obtained correlated with species of parasite, degree of parasitemia and time course of infection. This will be a continuing study.

Serial No. NIAID 133-H
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Monograph - "The Biology of the Primate Malarias."

Previous Serial Number: Same

Principal Investigators: Peter G. Contacos and William E. Collins

Other Investigators: McWilson Warren and G. Robert Coatney

Cooperating Units: Division of Research Services (Medical Arts and Photography Branch) and the Center for Disease Control

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	7/12			7/12
Professional	3/12			3/12
Other	4/12			4/12

Project Description:

Objectives: To prepare for publication as a monograph a comprehensive review of the biology of the malaria parasites of primates. Color plates are being prepared for inclusion in this publication.

Methods Employed: Data collected by our Laboratory on strains and species of simian, anthropoid and human malarias from Southeast Asia, Africa and South America are being reviewed and organized on the basis of speciation, host and geographical distribution, immunity, ecology, drug responses, etc. In association with the medical illustrator, color plates of erythrocytic stages of all the species of the primate malarias are being prepared. Data on the exo-erythrocytic and sporogonic development of these parasites is being organized by the staff of the Section on Primate Malaria in Chamblee for inclusion in this publication.

Progress: Color plates on the erythrocytic stages of all species of primate malaria have been completed including plates on the comparative aspects of *Plasmodium schwetzi*, *P. brasilianum*, *P. knowlesi*, *P. cynomolgi* and *P. inui* in man and non-human primates. Descriptive text material to accompany the plates and reviews of ecology and host and geographic distribution has been completed.

Significance to Bio-medical Research and the Program of the Institute:

Recent investigations on the malarias of the non-human primates has been intensive. These parasites are the best models we have available for laboratory research on many problems related to the biology of human malaria. A compilation of the available information on the malarias of all primates, including man, will be of great value to many laboratories, including our own, carrying out investigations in this field.

Proposed Course of the Project: Project completed.

Publications:

Coatney, G. R., Collins, W. E., Warren, McW., and Contacos, P. G.: *The Primate Malarias*. U. S. Department of Health, Education and Welfare, Public Health Service Monograph, 28 Chapters, 1971 (In Press).

Serial No. NIAID 133-I
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee-Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Administration and Research Planning for the Section

Previous Serial Number: None

Principal Investigator: Peter G. Contacos

Other Investigators: William E. Collins and W. A. Krotoski

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	29/12			29/12
Professional	8/12			8/12
Other	21/12			21/12

Project Description:

This project furnished technical, supervisory and administrative services to all research projects of the two Units of the Section, as follows:

a) Over-all planning and coordination of research in the Section.

b) Supervision over personnel, maintenance, and correspondence.

c) Requisitioning and supervision of equipment and supplies.

d) Preparation of reports, manuscripts, teaching materials.

e) Consultatory services to individuals, commercial organizations, non-governmental organizations and governmental agencies including liaison activities with other branches of the Public Health Service.

f) Reviewing, editing, and revising scientific and technical reports and manuscripts.

During the past year there have been several changes in assignment of personnel to the Section.

Dr. William A. Howard was appointed to the staff of the Unit on Human Malaria, Atlanta. Dr. Peter Briesch completed his two years of duty in the Unit on Human Malaria and was separated from the Public Health Service. Dr. Wojciech A. Krotoski was appointed Medical-Officer-in-Charge of the Unit on Human Malaria.

Consultative arrangements were maintained with Dr. Edmund Farrar of the Department of Preventive Medicine, Emory University, School of Medicine for the Unit on Human Malaria.

Cooperative studies continue with scientific groups within as well as outside the National Institutes of Health, especially with the Central America Malaria Research Station of the Center for Disease Control.

The research program of the Section and the participation of staff members in professional affairs outside of the NIH have continued to act favorably on the scientific community.

Honors and Awards:

Peter G. Contacos

Continues as Member, Expert Advisory Panel on Malaria, World Health Organization, reappointment April 7, 1971 for 5 years.

Continues as Associate Member, Commission on Malaria, Armed Forces Epidemiological Board.

Continues as Member, Interagency Board of U. S. Civil Service Examiners for North Georgia.

Participant in Malaria Courses of the Center for Disease Control, 1970-1971.

William E. Collins

Participant in Malaria Courses of the Center for Disease Control, 1970-1971.

Participant in two sections of the Second International Congress of Parasitology, Washington, D.C., September 1970.

Consultant, Central America Malaria Research Section, El Salvador, January 1971.

Publications:

Contacos, P. G.: Primate Malarias: Man and Monkeys. J. Wildlife Dis., 6: 323-328, October 1970.

Sodeman, T., Schnitzer, B., Durkee, T., and Contacos, P.: Fine Structure of the Exoerythrocytic Stage of *Plasmodium cynomolgi*. Science, 170: 340-341, October 1970.

Coatney, G. R., Collins, W. E., Warren, McW., and Contacos, P. G.: The Primate Malarias. U. S. Department of Health, Education and Welfare, Public Health Service Monograph, 28 Chapters, 1971 (In Press).

Serial No. NIAID 133-J
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effect of Heparin Anticoagulation on the Course of *P. knowlesi* Malaria in the Rhesus Monkey

Previous Serial Number: None

Principal Investigator: William A. Howard

Other Investigators: William E. Collins, Peter G. Contacos and Wojciech A. Krotoski

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	8/12			8/12
Professional	2/12			2/12
Other	6/12			6/12

Project Description:

Objectives: To confirm the reported antimalarial effect of heparin; to determine whether a coagulopathy develops in rhesus monkeys during *P. knowlesi* malaria; to determine whether heparin anticoagulation is of therapeutic benefit in prolonging longevity of rhesus monkeys infected with *P. knowlesi* malaria.

Methods Employed: Rhesus monkeys will be infected with *P. knowlesi* malaria by the bites of mosquitoes and randomly divided into heparinized and non-heparinized groups. Non-infected heparinized and non-heparinized controls will be included. Hematologic and coagulation parameters as well as parasite counts will be followed serially in each monkey.

Major Findings: Attempts to duplicate the previously reported effective anticoagulant doses of heparin in rhesus monkeys were unsuccessful as the animals repeatedly hemorrhaged internally. The heparin dosage and a route of administration have subsequently been revised.

Significance to Bio-medical Research and the Program of the Institute:

Consumption coagulopathy has been proposed as a terminal complication of acute falciparum malaria in man. Initial studies demonstrating coagulation factor depletion in human *Plasmodium falciparum* malaria and *P. knowlesi* malaria in monkeys consistent with a consumption coagulopathy have been disputed by other investigators. In terms of the implications these studies hold for using anticoagulant therapy as a treatment adjunct in acture falciparum malaria, additional investigation is warranted. Furthermore, the reported antimalarial effect of heparin needs confirmation and further investigation.

Proposed Course of the Project: The definitive study will now be initiated as the problems with anticoagulation of monkeys have been solved.

Serial No. NIAID 133-K
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Comparative Histological and Histochemical Studies on the Sporogonous Cycle of Malaria Parasites.

Previous Serial Number: None

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	16/12			16/12
Professional	8/12			8/12
Other	8/12			8/12

Project Description:

Objectives: To study (1) morphological or histological differences between the development of the parasite in mosquitoes showing different degrees of susceptibility to plasmodial infections; (2) histochemical demonstration of the relationship between the mosquito tissue and the parasite.

Methods Employed: (1) Histological: *Plasmodium* sp. are studied in laboratory bred mosquitoes (*Anopheles balabacensis balabacensis*, *A. maculatus*, and *A. freeborni*) fed on experimentally infected monkeys (*Macaca mulatta*). The sporogonic stages are obtained in paraffin sections of infected mosquitoes 0 through 21 days after ingestion of blood. Sections are stained with Giemsa or Hematoxylin; (2) Histochemical: (a) the localization of DNA is determined by the Feulgen Reaction; RNA is detected by staining with methyl-green pyronin with or without pretreatment with ribonuclease. (b) the detection of polysaccharides is carried out by staining sections with periodic acid Schiff, Bauer Feulgen staining methods, Best's carmine and Alcian Blue for acid mucopolysaccharides.

Major Findings: From the histological studies it appears that ookinetes which are formed after the ingestion of an infective blood meal migrates to the gut wall at about 48 hours after feeding. Small oocysts are found on the external gut wall 72 hours after ingestion of blood. Some developing oocysts, however, become impacted within the gut wall of some mosquitoes. These oocysts in apparently an intracellular position develop only partially. Degenerative changes, during the different stages of development of oocysts are characterized by the break-up of the orderly distribution of DNA and RNA and coagulation of the sporoplasm with the formation of amorphous DNA or RNA droplets. Oocysts containing such inclusions may degenerate partially or totally with formation of vacuoles and deposition of refractile pigments on the surface.

RNA positive reactions are (1) particularly marked in the cytoplasm of oocysts, stomach epithelial cells, oocysts and nutritive cells; (2) less marked in the cytoplasm of salivary gland cells, malpighian tubules cells, and connective tissue cells; and (3) absent in muscle cells. DNA positive reactions are seen in oocysts and all nuclei of mosquito tissue. Cytochemical tests indicated the absence of glycogen from the oocyst cytoplasm. A positive reaction was found only in the oocyst wall, epithelial stomach cells, basement membranes and haemolymph. Evidence of the presence of acid mucopolysaccharides in developing and mature oocysts was confirmed by applying the Alcian Blue staining method.

Significance to Bio-medical Research and the Program of the Institute:

The incidence of malaria in its many ecological environments is primarily dependent on those factors which bring a suitable mosquito vector in contact with the susceptible host. Although it has been recognized for many years that not all anophelines are capable of transmitting malaria, the reasons for this are, in many instances, unclear. These studies will not only assist in an understanding of the reasons for mosquito susceptibility to malaria, but more importantly, what makes a species a vector.

Proposed Course of the Project: (1) The study of the differential structure and reactions of mosquito tissues which may affect the development of malaria parasites; (2) to investigate physiological and biochemical factors in anopheline mosquitoes susceptible to infection but with varying degrees of vector potential and their probable importance in the transmission of malaria.

Serial No. NIAID-134
1. Parasitic Diseases
2. Primate Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathogenesis of the malarial paroxysm

Previous Serial Number: None

Principal Investigator: Franklin A. Neva

Other Investigators: W. A. Krotoski and W. A. Howard

Cooperating Units: Department of Justice, Bureau of Prisons, Federal Penitentiary, Atlanta, Georgia; Section on Medical Parasitology of Laboratory of Clinical Investigation

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	15/12			15/12
Professional	3/12			3/12
Other	12/12			12/12

Project Description:

Objectives: (1) to define, with greater precision than has been done previously, the time relationship of parasite development, to onset and subsequent elements of the malarial paroxysm and (2) to determine causative factors in the genesis of fever and other manifestations of the malarial paroxysm.

Methods Employed: Patients with naturally occurring or induced malaria are studied whose infections exhibit a highly synchronized cycle of parasite development and/or a well synchronized cycle of clinical signs and symptoms. By use of an indwelling venous catheter, blood samples are obtained at frequent intervals before, during, and after a paroxysm and rectal temperatures are also recorded. Thin smears for parasite differentials have been taken at 15 minute intervals, and specimens for other determinations (WBC, platelet count, etc.) at least frequent intervals. Serum complement levels are measured with a standardized 50 percent hemolytic end point technique.

Parasite differential counts are performed independently on 100 parasites by several different observers.

Major Findings: The parasite differentials on the first 6 or 8 patients studied are still being analyzed and re-counted in an effort to standardize the procedures and establish a uniform classification of different forms of the parasites. Several of the early patients studied were not optimum cases for study in that the parasite cycle was not well synchronized or the collection of specimens did not completely span the paroxysm. From comparative differential counts of parasites it was decided that only thin films would be used, at least 100 parasites would be counted, and that parasites counted should not be from extreme edges of the film. Preliminary data suggest that rupture of mature schizonts can be evaluated in terms of two functions: (1) actual decrease in percentage of mature schizonts and (2) first appearance and increase in percentage of young ringform parasites. Two of the first three patients in whom complement levels were determined have exhibited significant changes in complement levels.

Significance to Biomedical Research and the Program of the Institute:

Program of the Institute: Although a tremendous background of information has developed concerning the pathogenesis of fever following administration of pyrogens to experimental animals, the significance of these findings to the cause of fever in human infections is not at all certain. Furthermore, while fever as a consequence of malarial schizogony has been a recognized and accepted phenomenon for years, a precise definition of the sequence of events and the time periods involved is not available in the literature. With the background of experimental data now available it seems appropriate to seek information on the events and cause of fever in as time-honored and common human disease as malaria. Many of these studies can be carried out as a "by-product", or in association with other research on malaria in human volunteers which is conducted in the laboratory. The long term interest and research experience in fever as a basic feature of disease in the Laboratory of Clinical Investigation can be utilized as approaches for further study are developed. Information that may be derived from this project about the cause of fever in a human disease may be applicable to other diseases characterized by fever.

Proposed Course of Project: This project is still in a beginning stage - a standard protocol is still in the process of development. A methematical approach may be needed to derive an arbitrary zero point of maximum schizont rupture from changes in values of mature schizonts and early ringform parasites. If a reasonably consistent latent period between maximum schizont rupture and onset of paroxysm can be established, it would be helpful in selecting a time for attempting to demonstrate a transferable circulating pyrogen as a cause of malarial fever.

Serial No. NIAID 134-A
1. Parasitic Diseases
2. Primate Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of Antimalarial Drugs and/or Drug Regimens.

Previous Serial Number: Same

Principal Investigators: Peter G. Contacos and Franklin A. Neva

Other Investigators: W. A. Krotoski and W. A. Howard

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	15/12			15/12
Professional	2/12			2/12
Other	13/12			13/12

Project Description:

Objectives: To evaluate standard antimalarial agents and/or new drugs known to have antimalarial activity and to develop acceptable regimens for their use against human malaria.

Methods Employed: Healthy inmate volunteers at the U. S. Penitentiary, Atlanta, Georgia, are screened before they are admitted to the project. Volunteers are under 24-hour care and supervision of the resident investigators. For the evaluation of chemotherapeutic agents against malaria, volunteers are usually exposed to infection by the bites of infected mosquitoes and only occasionally by the intravenous inoculation of homologous parasitized blood. For studies on the prophylactic and/or suppressive effect of drugs, volunteers are medicated before exposure to infection. For studies on the therapeutic and/or radical curative effects of drug, volunteers do not receive drugs until patent infection is demonstrated. Volunteers are observed for a minimum of six months although follow-up has been extended at times for up to 18 months.

Major Findings: Antimalarial Efficacy of Lincomycin Analogs: The antimalarial activity of Cleocin® (7-chlorolincomycin - clindamycin) has been tested against two species of human malaria. Three infections of vivax malaria have been treated with 450 mg every 6 hours for 7 consecutive days. Parasite clearance occurred in 96 to 120 hours after initiation of therapy. Clearance of clinical manifestations (fever) was in 48-72 hours. Two infections of ovale malaria were also treated with the following results: parasite clearance of 120 to 168 hours and clinical clearance of 120 to 144 hours. This drug is undoubtedly "slow-acting" against at least vivax and ovale malaria. Trials with falciparum malaria are currently under way.

Significance to Bio-medical Research and the Program of the Institute:

The need for additional chemotherapeutic methods and drugs against malaria is becoming more actue with the increased appearance of strains of falciparum malaria resistant not only to all commonly used synthetic drugs but also to quinine. Because of our commitments, whether military or civilian, in countries where malaria is still prevalent, more effective chemotherapeutic methods for protecting our wholly susceptible people from malaria are required.

Proposed Course of the Project: To continue the study of Cleocin® and hopefully another lincomycin analog known as U24-729A (N-dimethyl-4'-pentzl-7-chlorolincomycin) when and if it is approved for licensure by FDA. This latter analog had significantly greater antimalarial activity against malaria in monkey than did Cleocin®.

Serial No. NIAID 134-B
1. Parasitic Diseases
2. Primate Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in Human Malaria

Previous Serial Number: Same

Principal Investigators: Peter G. Contacos and Wojciech A. Krotoski

Other Investigators: William A. Howard, Franklin A. Neva and William E. Collins

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	47/12			47/12
Professional	7/12			7/12
Other	39/12			39/12

Project Description:

Objectives: To study the human malarias in volunteers for clinical manifestations, parasitologic course, pathophysiology of the infection, immunologic response of the host, and effect of standard antimalarial drugs. Particular emphasis is placed on characterization of newly isolated strains by determining their sensitivity to antimalarials and normal relapse patterns which can be expected after non-curative treatment. Also to study the human malarias in non-human primate hosts.

Methods Employed: Healthy inmate volunteers at the U. S. Penitentiary, Atlanta, Georgia, are screened before they are admitted to the project. Volunteers are under the care of the resident investigators. They are exposed to infection either by the bites of infected mosquitoes or by the intravenous inoculation of homologous parasitized blood. They submit freely to necessary laboratory procedures and receive antimalarial or other medications only as prescribed by the resident investigators. Participation in any one project may range for 6 - 18 months depending on the nature of the project. Standard suppressive and curative regimens of antimalarial treatment are administered and the patient observed for immediate clinical and parasitological response and for longer periods of time to determine effect of the infection on the formed elements of the blood and on coagulation and other blood factors. Serum is obtained at various intervals and frozen for subsequent evaluation of antibody titers. For the latter tests, fluorescent antibody determination and separation of serum protein by various electrophoretic methods will be utilized.

Major Findings:

I. Studies on human malaria in human volunteers.

Studies on four recently isolated strains of Plasmodium vivax.

1. Panama strain: Primary isolation of this strains was in an owl monkey. Ten volunteers exposed to infection by the bites of heavily infected mosquitoes, developed patent infections in 12 to 17 days with a mean of 13.5 or median of 13 days. Eight experienced 1 relapse; six exerienced 2 relapses; three experienced 3 relapses. The first relapses occurred at 94 to 226 days; the second relapses at 148 to 246 days; and the third relapses at 251 to 298 days. This strain of vivax malaria was observed to be sensitive to recommended dosages of chloroquine, pyrimethamine, or quinine.

2. Salvador I strain: Primary isolation of this strain was in an owl monkey. Ten volunteers exposed to infection by bites of heavily infected mosquitoes developed patent infections in 12 to 16 days with a mean of 14.5 or median of 15 days. None relapsed during the first 180 days at which time five volunteers elected not to extend and were given radically curative therapy. Of the remaining five volunteers, only two relapsed at 200 and 229 days after exposure to infection. This strain was observed to be sensitive to the recommended dosages of chloroquine, chlorguanide, pyrimethamine, and quinine.

3. Salvador II strain: Primary isolation was in an owl monkey. Nine volunteers exposed to infection by bites of heavily infected mosquitoes developed patent infections in 9 to 16 days with a mean of 12.7 or median of 13 days. Four volunteers received radical curative therapy at 180 days with no relapses. Of the remaining five volunteers, three experienced 1 relapse; one experienced a second relapse. First relapse was at 172 to 262 days; the second relapse was at 203 days. This strain was observed to be sensitive to the recommended dosages of chloroquine, chlorguanide, or quinine.

4. NICA strain: This strain from Nicaragua was isolated in an owl monkey. To date, only two volunteers have been infected with prepatent periods of 9 and 13 days. No relapse pattern studies have been initiated. This strain is sensitive to chloroquine.

II. Studies on human malaria in non-human primates.

In our continuing studies concerned with the experimental infection of New World monkeys with human malaria, the major findings for the year can be summarized as follows:

1. Infection of owl monkeys (Aotus trivirgatus) with two strains of Plasmodium falciparum (one from Panama and one from South Vietnam). Both isolates were lost. Subinoculation of the Panama isolate into several monkeys resulted in no infection.

2. Infection of owl monkeys with three new isolates of Plasmodium vivax (Salvador II, South Vietnam III, and Nica). The origin of the NICA strain is Nicaragua.

3. Transmission of the Salvador I, Salvador II, and Nica strains of Plasmodium vivax from the owl monkey back to man was by the bites of infected mosquitoes.

4. The Panama strain of Plasmodium vivax was transmitted to owl monkey from man by the intrahepatic inoculation of sporozoites from mosquitoes infected on man.

Significance to Bio-medical Research and the Program of the Institute:

Malaria remains one of the major world-wide causes of morbidity and mortality. With our increasing international commitments in areas endemic for malaria, particularly in Southeast Asia, the number of American personnel, military and civilian, exposed to malaria in such areas is increasing. In recent years, reports of Americans returning to this country and developing malaria with onset in this country are also increasing. The imported malarias have the potential for infecting local anopheline mosquitoes, and it is necessary to study some of these imported strains of malaria to determine their clinical manifestations, the ease with which they can be transmitted, and their sensitivity to the standard antimalarial drugs.

Current knowledge concerning human quartan malaria is particularly meager. Experimental transmission of this malaria has been accomplished only rarely, yet continued natural transmission in areas where malaria eradication programs are under way constitutes a major source of new infections. Relapse patterns of this species are not known. The availability of a strain of P. malariae for study provides an excellent opportunity to contribute essential knowledge concerning this human malaria.

The fact that small non-human primates can be infected with human malaria could obviate the necessity of employing volunteers for certain aspects of malaria research programs such as for the purpose of infecting mosquitoes for transmission studies. Most importantly, the infection of such small experimental animals with human malaria should increase the ability of even small research centers to study the biology and drug susceptibility of new and/or old isolates of human malarias.

Proposed Course of the Project: To continue the study of imported malarias with emphasis on their potential for spread in this country, and to continue investigations on pathophysiology and immunology of human malarias. Special emphasis will be given to the infection of non-human primates with all of the strains of human malarias in our possession.

Publications:

Coatney, G. R., Collins, W. E., Warren, McW., and Contacos, P. G.: The Primate Malarias. U. S. Department of Health, Education and Welfare, Public Health Service Monograph, 28 Chapters, 1971 (In Press).

Contacos, P. G.: Primate Malarias: Man and Monkey. J. Wildlife Dis., 6: 323-328, 1970.

Serial No. NIAID 67C

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on arboviruses

Previous Serial Number: Same

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Lloyd Rozeboom and Dr. Jacques Saugrain

Cooperating Units: School of Hygiene and Public Health, Johns Hopkins University, Baltimore; Institut des Recherches Medicales, Papeete, Tahiti

Man Years:	NIAID staff
Total:	8/12
Professional:	8/12
Others:	0/12

Project Description:

Objectives

1. To study the epidemiology of arboviruses in the simple ecologic environment of Pacific Islands.

2. To study the relative susceptibility of various species of mosquitoes to infection with dengue viruses.

3. To improve laboratory methods for the detection of dengue viruses and their antibodies.

Methods Employed

Studies were continued on the parameters of dengue virus replication and plaque production in LLC-MK_2 and a continuous line of *Aedes albopictus* cells. Studies also were carried out on the replication of dengue viruses injected intrathoracically and fed to various vector and non-vector species of mosquitoes. A prospective serological study was initiated in French Polynesia in an attempt to determine if dengue infection is now endemic there.

Major Findings

Aedes aegypti, Aedes albopictus, and Aedes polynesiensis, were successfully infected by intrathoracic inoculation with prototype strains of dengue types 1, 2, and 4 which had not previously been passed in cell cultures or mice. Virus titers obtained in individual mosquitoes exceeded 10,000 ID50 for types 1 and 4, and 100,000 ID_{50} for type 2 as measured in cell cultures. However, virus titers of 100,000,000 ID_{50} per mosquito were demonstrated for type 2 by intrathoracic inoculation of susceptible species of mosquitoes. No differences were detected among these three mosquito species in the percentage of mosquitoes infected or in virus titers achieved, but Armigeres and Culex mosquitoes inoculated at the same time with the same doses of virus became infected less frequently and virus titers achieved in infected specimens often were lower than was the case for the Aedes. The Aedes species have not as yet been infected successfully by feeding on virus suspensions.

Comparative titrations of dengue viruses in cell cultures and by intrathoracic inoculation of susceptible species of mosquitoes demonstrated that, for the three serotypes mentioned above, mosquito inoculation was 10 to 1000 times more sensitive an indicator of the presence of virus.

An inhibitor was found in the supernatant fluid of dengue infected cell cultures which considerably reduced the titer of the culture. Virus yields were significantly improved by discarding this fluid and washing the cells before harvest.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are increasing in significance as a cause of morbidity and mortality in Southeast Asia and Pacific Islands - probably because of rapidly increasing urbanization and travel in that geographic area. Mosquito vectors of dengue viruses are ubiquitous in the same area, including many islands which belong to, or which are controlled by, the United States. Obviously, further understanding of the epidemiology and vectors of dengue would be of value.

If the intrathoracic inoculation technique fulfills the promise shown in experiments so far completed, it will provide, for the first time, the sensitive laboratory tool needed to carry out careful studies of the pathogenesis of dengue infection in man and lower primates and dengue hemorrhagic fever in man. Thus far, such studies have been handicapped by the absence of a sensitive method of detecting relatively small amounts of unmodified dengue virus.

Arboviruses are important causes of human disease in both temperate and tropical areas. Aside from dengue, studies on arboviruses on Pacific islands should yield information not easily obtainable in more complex ecologic settings.

Proposed Course of the Project

It is anticipated that most of the effort on this project next year will be devoted to a study of the quantitative aspects of dengue virus replication in vector and non-vector mosquito species and the use of the intrathoracic injection technique for detection of dengue viruses.

Of course, should an outbreak of arbovirus disease occur on a suitable island, new field studies would be undertaken. Because of their relative isolation, the frequency of infection on such islands is much less than in continental areas and opportunities for study are largely a matter of chance.

Honors and Awards:

Dr. Rosen was elected to membership in the Infectious Diseases Society of America. He continued to serve as a member of the Virus Diseases Panel, U.S. Japan Cooperative Medical Science Program; as an Associate Member, Commission on Viral Infections, Armed Forces Epidemiologic Board, as a member of the Dengue Task Force of the same Commission; as a member of the Arboviruses Committee, NIAID; as a member of the ad hoc Enterovirus Advisory Group, NIAID; and as a member of the Affiliate Graduate Faculty, University of Hawaii (School of Public Health, College of Health Sciences and Social Welfare and Department of Microbiology, College of Arts and Sciences).

Dr. Rosen organized and chaired a workshop on "The Pathogenesis of Dengue Hemorrhagic Fever" held in San Francisco under the sponsorship of the U.S. Panel on Viral Diseases of the U.S. Japan Cooperative Medical Science Program and also chaired a panel discussion on the "Development of rabies collaborative studies and control programs in Southeast Asia" at a Working Conference on Rabies held in Tokyo, Japan held under the joint sponsorship of the U.S. and Japanese Viral Diseases Panels. He was asked (and agreed) to arrange the program for a conference on the "Pathogenesis of Arboviral Infections" to be held in Honolulu under the same joint sponsorship in September, 1971.

Publications:

Rosen, L., Melnick, J.L., Schmidt, N.J., and Wenner, H.A.: Subclassifications of enteroviruses and ECHO virus type 34. Arch. Ges. Virusforsch., 30:89-92, 1970.

Rosen, L.: Reoviruses. In Standardized Methods for Veterinary Microbiology, to be published by the National Academy of Sciences (in press).

Rosen, L.: Reoviruses. In Hubbert, W.T., McCulloch, W.F., and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man - 6th Edition, (in press).

Saugrain, J., Rosen, L., Outin-Fabre, D., and Moreau, J.P.: Une recent epidemie d'arbovirose du type dengue a Tahiti comparaisons avec l'epidemic de 1964. Bull. Soc. Path. Exot., (in press).

Serial No. NIAID 67D

1. Laboratory of Parasitic
   Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the natural history of *Toxoplasma gondii* and the mechanism of its transmission to man.

Previous Serial Number: Same

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. D. Carleton Gajdusek

Cooperating Units: NINDS, Study of Child Growth and Development and Disease Patterns in Primitive Cultures; Hawaii State Department of Agriculture; Hawaii State Department of Public Health.

Man Years:	NIAID staff
Total:	12/12
Professional:	12/12
Others:	

Project Description:

Objectives

To determine the method, or methods, by which man becomes infected with *T. gondii*.

Methods Employed

Working hypotheses developed from information obtained during the past two years are being tested by experimentation and field observation, taking advantage of unique epidemiologic situations that occur on islands in the Pacific Ocean. The hypotheses being tested are: 1) cats are the definitive host of *T. gondii*; 2) certain small vertebrate animals, normally the prey of cats, are obligatory intermediate hosts; 3) in the Pacific area, man usually becomes infected with *Toxoplasma* by ingesting oocysts; 4) invertebrate transport hosts are involved in maintaining the life cycle of the parasite and in its transmission to man.

Major Findings

Experimental

Cats. It has been well established through experimentation in our laboratory, as well as in others, that susceptible cats fed tissues of chronically infected mice containing the cyst stage of Toxoplasma routinely excrete infectious Toxoplasma oocysts in their feces. The oocyst stage, which is highly resistant to environmental factors, is shed for an average of 10 days following a 4-5 day prepatent period. The Toxoplasma oocyst cycle has not been demonstrated in animals other than Felidae, although members of several families have been tried.

During the past year a number of experiments on domestic cats have been conducted to provide detailed information on antibody response and immunity in relation to oocyst production. These studies involved exposure of cats to the cyst, trophozoite, or oocyst stage of the parasite, and included use of various small animals as intermediate hosts.

In cats following ingestion of Toxoplasma cysts, dye-test antibody usually became detectable within 7-10 days. Therefore specific antibody was present in the serum during the latter part of the oocyst excretion period. Four months after initial infection, cats were completely immune to challenge with cysts but were only partially immune (long prepatent and short patent periods) to challenge at 7-8 months. At this time antibody was at very low levels and did not increase in titer following re-exposure.

Oocyst excretion was observed in about 50% of cats fed mice or doves acutely ill with toxoplasmosis and presumed to harbor only the trophozoite stage of the parasite. However the prepatent period was long, the patent period was short, and antibody appeared later and remained at lower levels than in cats exposed to cysts.

Cats were relatively resistant to infection with the oocyst stage of Toxoplasma and did not respond serologically unless given large numbers (>20,000). Of 26 cats exposed orally to various numbers of oocysts, only 50% responded serologically, and only two developed patent infection. In these two, prepatent periods were extremely long and patent periods were short.

In summary, experimental data suggest that Toxoplasma is maintained in nature through a cyst-oocyst-cyst cycle, or possibly a trophozoite-oocyst-trophozoite cycle, involving the cat as a definitive host and small rodents and birds as intermediate hosts.

Transport hosts. Invertebrates feeding on cat feces might serve as transport hosts and vectors of the Toxoplasma oocyst. Filth flies, certain terrestrial molluscs, and cockroaches are likely candidates. Results of fly experiments have already been reported. A veronicellid slug and the American cockroach were chosen for experiments because they are known to be intermediate hosts of helminthic parasites shed in cat feces. The Madera roach was also used because its habits are similar to the American roach. Experiments on the slugs were not successful because in captivity these animals would not feed on cat feces. However the cockroaches readily consumed infectious cat feces and in preliminary experiments Toxoplasma was isolated from digestive tracts and feces of cockroaches for as long as 4-5 days after exposure.

Field studies on cats and rats. The project on the prevalence of Toxoplasma antibody and oocysts in stray or unwanted cats on Oahu, Hawaii was continued throughout the year. Of 451 cats examined, viable Toxoplasma oocysts were found in the feces of 4. Toxoplasma antibody was relatively uncommon in cats under 5-6 months of age, suggesting that transmission in these animals might be occurring through an intermediate host rather than from contact with feces. This study has provided the first documentation of Toxoplasma in the feces of cats in their natural habitat.

Sera from 578 wild rats trapped on three Pacific islands have been tested for Toxoplasma antibody. Cats were evident on two islands but were not present on the other. On the islands where cats were present, an average of 4% of the rats had Toxoplasma antibody, but there was clustering of positives as high as 20% in some areas. All of 190 rats tested from the "catless" island, however, were completely negative.

Seroepidemiology, human

Hawaii. The prevalence of Toxoplasma antibody among ethnic groups was investigated on the island of Hawaii. This study involved 215 high school students living in a stable rural community. The majority had lived in the community and frequently in the same house all of their lives. A blood specimen was collected from each student and information was obtained on cultural traits including food habits, socioeconomic status, sanitation in the home, and contact with animals. Of five distinct ethnic groups involved in the study, the prevalence of Toxoplasma antibody ranged from a low of 14% in Japanese to a high of 67% in Portuguese. Epidemiologic information revealed that the groups with the lower prevalences of antibody had much less contact with cats and lived under better sanitary conditions than the groups with the higher prevalences. The consumption of raw or rare meat did not appear to be related to antibody prevalence.

New Guinea. Sera collected by Dr. D. Carleton Gajdusek, NINDS, from primitive natives inhabiting remote isolated areas of New Guinea were tested for Toxoplasma antibody. Felids are not native to New Guinea, and were not present in the areas inhabited by the people sampled. Only 6 of 641 sera tested contained antibody - an extremely low prevalence compared to natives of other Pacific islands.

Namoluk Atoll. Detailed seroepidemiologic studies are being conducted on a small isolated atoll in the Western Pacific where the natural fauna is relatively simple and there are few domestic animals. Blood specimens have been obtained from about 70% of the 300 human inhabitants, 75% of 35 cats and all of 27 pigs. There had been no dogs on the island since 1963. Specimens have also been collected from 220 wild rats, the only wild mammals on the island except for fruit bats. Seventy percent of the people had Toxoplasma antibody, many with high titers. The prevalence of antibody in the cats, pigs, and rats was 85%, 20%, and 4%, respectively. Toxoplasma antibody was present in a large proportion of children who were born after 1963 and had never been away from Namoluk. This finding along with finding antibody in rats, cats, and pigs born on the island within the past 1-3 years, rules out the dog as a definitive host of Toxoplasma. Observations are being made on the habits of the people and animals that might relate to transmission of Toxoplasma.

Significance to Biomedical Research and the Program of the Institute

Human toxoplasmosis is recognized as a medical problem throughout the world but the principal method (or methods) by which man becomes infected is unknown. Obviously, new information on this topic would be of considerable interest. Hawaii and other Pacific islands offer unique opportunities to gain insight into the life history and transmission of Toxoplasma gondii.

Proposed Course of Project

Efforts will continue to be directed towards testing the working hypotheses mentioned previously (see Methods Employed).

To date, data from experiments and field investigations suggests that cats would seldom develop a patent infection (excrete oocysts) from exposure to Toxoplasma oocysts in nature. Rather, the evidence indicates that the cyst stage and possibly the trophozoite stage are essential for the prepetuation of Toxoplasma (involving intermediate hosts and possibly transport hosts). Additional experiments on intermediate hosts are being planned and will include observations on the responses of wild rats and mice, certain species of wild birds, and geckos to exposure to Toxoplasma oocysts. More experiments on cockroaches as transport hosts will also be undertaken.

Seroepidemiologic studies on man and lower animals will be continued for the purpose of comparing prevalences of Toxoplasma antibody between areas where cats occur and areas where cats are absent. The Caroline Islands provide unique situations in this regard. Small islands or islets inhabited by man, cats, rats, and few other mammals are located near other islands where rats are the only mammal present. Rats will be used as indicators of the occurrence of Toxoplasma in these situations. Seroepidemiologic studies comparing the prevalence of toxoplasmosis in man in the presence and absence of cats are being continued in New Guinea and are being initiated in the Cook Islands.

The project on stray cats on Oahu will be continued for the remainder of 1971. Evidence to date suggests that there is a seasonal fluctuation in the incidence of Toxoplasma infection in cats and isolations of Toxoplasma from cat feces will increase from July through October.

The public health importance of toxoplasmosis on most Pacific islands where the prevalence of infection is very high is not known. It is hoped that useful information can be obtained from studies of human disease on Truk and Namoluk, Caroline Islands and perhaps in other locations such as Samoa and the Cook Islands.

Honors and Awards:

Dr. Wallace continued to serve as Assistant Clinical Professor of Public Health and Assistant Clinical Professor of Tropical Medicine and Medical Microbiology in the University of Hawaii Schools of Public Health and Medicine, respectively.

Publications:

Wallace, G.D.: Experimental transmission of *Toxoplasma gondii* by filth-flies. *Amer. J. Trop. Med. Hyg.*, 1971 (In press)

Wallace, G.D.: Isolation of *Toxoplasma gondii* from the feces of naturally infected cats. *J. Infect. Dis.*, 1971 (In press)

Serial No. NIAID 67E

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on seasonal gastroenteritis at Clark Air Base, Philippine Islands.

Previous Serial Number: Same

Principal Investigator: Dr. Andrew G. Dean

Other Investigators: Dr. Robert Couch

Cooperating Units: Department of Microbiology, Baylor College of Medicine, Houston, Texas

Man Years:	NIAID staff
Total:	5/12
Professional:	5/12
Others:	

Project Description:

Objectives

Further microbiologic and human volunteer studies of the epidemic seasonal gastroenteritis which occurs at Clark Air Base were carried out. Routine causes of diarrheal illness had been previously excluded and attention was focused on *Escherichia coli*, bacteriophages, known viruses, and filtrable agents affecting human volunteers, as possible causes of the 1969 epidemic.

Methods Employed

Escherichia coli were isolated from stools of patients in the 1969 epidemic, in which an estimated 5,000 of the 35,000 Americans at Clark Air Base had gastrointestinal symptoms during March through July, 1969. These strains were used to make KO antisera in rabbits and a search was made for serologic relationships between *E. coli* from different individuals in the epidemic. *E. coli* from the patients were tested for enterotoxin production by inoculation into ileal loops of adult rabbits. A strain of *E. coli* from a healthy individual at Clark Air Base was used to detect bacteriophage in the stools of patients by the plaque method. Stools from the epidemic preserved at -70°C were studied virologically in human fetal kidney cell culture and in newborn mice. After extensive safety testing, supernatant fluids of centrifuged diarrheal stool suspensions were given orally to volunteers in Houston in an attempt to transmit the illness.

Major Findings

The strains of *E. coli* from 11 patients were found to be serologically diverse, and none consistently dilated rabbit intestinal loops. Bacteriophages affecting the indicator strain of *E. coli* were found in eight of ten patients' stools, but also in the same percentage of stools from healthy control individuals, at similar titers. From 48 acute stool specimens, only one adenovirus and two agents pathogenic for infant mice were isolated. Five 20% stool suspension supernates did not produce disease in 22 volunteers in doses of 2 to 22 milliliters per man.

Significance to Biomedical Research and the Program of the Institute

Careful study of this large epidemic of gastroenteritis using techniques for detection of virtually all known causes of diarrhea consistent with the epidemiologic picture, has produced no explanation for the illness. This not only illustrates the inadequacy of present knowledge of diarrheal disease, but, since the epidemics are recurring annually, provides an opportunity for further research. The fact that approximately 20% of the acute cases develop prolonged symptoms and laboratory findings indistinguishable from mild tropical sprue gives added meaning to the search for the cause of the epidemic.

Proposed Course of the Project

Since the principal investigator will be absent from September, 1971 to June, 1972, no further field work is planned for the near future. An attempt is being made to develop more sensitive methods for detecting bacterial enterotoxins, and if successful, these will be applied to material preserved from the 1969 epidemic.

Honors and Awards:

Dr. Dean continued to serve as Assistant Clinical Professor of Tropical Medicine and Medical Microbiology in the School of Medicine, University of Hawaii.

Publications:

None

Serial No. NIAID 67F

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on competition between *Aedes albopictus* and *Aedes polynesiensis* in nature.

Previous Serial Number: Same

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Lloyd Rozeboom, Dr. William Reeves, Mr. Gaston Pichon, and Dr. Jacques Saugrain

Cooperating Units: School of Hygiene and Public Health, Johns Hopkins University, Baltimore; School of Public Health, University of California, Berkeley; Institut des Recherches Medicales, Papeete, Tahiti.

Man Years:	NIAID staff
Total:	4/12
Professional:	4/12
Others:	0/12

Project Description:

Objectives

Aedes polynesiensis is a member of the *scutellaris* group of mosquitoes and the major vector of human filariasis (*Wuchereria bancrofti*) in Polynesia and parts of Melanesia. It is impractical to control this species by conventional means because of the great diversity of its small breeding sites and the fact that the adults do not rest in houses. *Aedes albopictus* is a related species of mosquito which does not occur naturally in the same geographic area as *A. polynesiensis*. It is completely refractory to infection with human filariae. Observations on *A. albopictus* in areas where it coexists with other members of the *scutellaris* group suggest that if *A. albopictus* were deliberately introduced into an area where a *scutellaris* species occurred, the latter might be greatly reduced in numbers, or even eliminated. Competition between both larval and adult forms of *A. albopictus* and *A. polynesiensis* has been studied intensively in the laboratory and these data also suggest that *A. albopictus* might greatly reduce the population of *A. polynesiensis* if the former were introduced into an area where the latter occurred. The objective of this project is to study the effect of such an introduction on an isolated island which harbors a natural population of A. polynesiensis.

Methods Employed

A heavily wooded coral atoll with an abundant A. polynesiensis population was selected as the locale for the experimental introduction of A. albopictus. Baseline population estimates of A. polynesiensis were made three times at each of a number of selected sites on the island at three-month intervals prior to the introduction of A. albopictus. Three strains of A. albopictus were released at widely separated localities on the island in January-February, 1970. One strain consisted of adults reared directly from eggs deposited by wild females on Oahu, Hawaii. The second consisted of adults reared from eggs from a laboratory colony originally started with specimens from Oahu, and the third consisted of adults reared from eggs from a laboratory colony originally started with specimens from Poona, India. It was estimated that between 3000 and 6000 females (and a comparable number of males) of each strain were released. Many of the females were given a blood meal in the laboratory prior to their release. Follow-up visits were made to the atoll in May and August, 1970 and February, 1971 to assess the fate of the liberated species.

Major Findings

The first visit (three months after release) was of short duration (4 days) and was made by subprofessional personnel to determine if A. albopictus had survived. Although extensive quantitative data were not obtained, both larvae and adults of A. albopictus were found in the immediate vicinity of each of the three release areas - the only sites sampled. Both adults and larvae appeared to be more abundant at the site of release of the Hawaiian wild strain than at the other two sites. Albopictus larvae were found in 11 (69%) of the 16 breeding sites containing mosquito larvae in the three areas.

The second visit (six months after release) offered an opportunity for the collection of a greater amount of quantitative data but was handicapped by the very dry condition of the island and the resultant low larval and adult population. At this time, no A. albopictus adults were found at the site of release of the Indian laboratory strain or in nearby areas. One of 13 larval samples collected there, however, did contain this species. A. albopictus adults were found at both other release areas, at various places between them, and beyond each release site in the opposite direction for at least 500 meters. In all, the species was found over an area at least five kilometers wide. Surprisingly, A. albopictus did not appear more abundant in the immediate vicinity of its release than it did at relatively distant sites. In the five-kilometer-wide area mentioned above, approximately 3 percent of the Aedes females captured on human bait were A. albopictus. A. albopictus adults appeared to be somewhat more abundant in that part of the area where the Hawaiian wild strain had been released than they did where the Hawaiian laboratory strain had been liberated. Five (22%) of 13 larval samples collected in the five-kilometer-wide area contained A. albopictus larvae.

At the third visit (one year after release) the adult Aedes population was greater than at the previous visit but adult A. albopictus were comparatively less common, comprising only 1 percent of Aedes females captured in the five-kilometer-wide area noted above. No A. albopictus was found among 954 female Aedes captured at the site of release of the Indian strain nor were A. albopictus larvae found in 11 larval samples obtained there. At this visit, A. albopictus adults definitely were less abundant at the site of release of the Hawaiian wild strain than there were in an area of large trees beyond the site of release of the Hawaiian laboratory strain. Fifteen (25%) of 61 larval samples collected in the five-kilometer-wide area contained A. albopictus larvae.

In summary, A. albopictus has persisted on the atoll for one year, although one of the three strains liberated appears to have died out. Surprisingly, A. albopictus did not appear to favor artificial containers as larval breeding sites nor the one area on the island inhabited by the few people and domestic animals present (the area where the Hawaiian wild strain was released). At the last visit it did appear more abundant in a well-protected forest of large trees - the area on the atoll which most resembled that of a volcanic island. In retrospect, it appears that because of the sporadic nature and short duration of rainfall on the island, and the high evaporation rate, considerable time elapses between each mosquito generation. Although the ultimate fate of A. albopictus on the island is still unknown, the presence of relatively large numbers of larvae of the species at the time of the last visit suggests that it will persist. Whether or not it eventually will have a significant impact on the A. polynesiensis population is still unknown.

Significance to Biomedical Research and the Program of the Institute

If the experiment is successful, it will provide a means of controlling, or even eradicating, human filariasis in the entire endemic area in Polynesia and parts of Melanesia at little or no cost. The experiment also has significance in that it provides an opportunity to make observations on the population dynamics of a newly introduced mosquito species under controlled conditions. In view of the current interest in the control of vector species by biologic (rather than chemical) methods, the data obtained from this project should be of considerable value.

Proposed Course of the Project

Follow-up visits to the atoll will be continued to determine the ultimate outcome of the experiment but the frequency of visits probably will be reduced to yearly intervals unless a more rapid change in the A. albopictus population is observed. In an attempt to determine the effect of an A. albopictus introduction on A. polynesiensis more quickly, it is planned to carry out a second introduction of A. albopictus on Rarotonga, Cook Islands. Rarotonga is a volcanic island which should be a more favorable habitat than an atoll for A. albopictus and also is an area where filariasis transmitted by A. polynesiensis is endemic. Because of the greater accessibility of this island, it is planned to release larger numbers of A. albopictus over a longer period of time than was done on the atoll and also to carry out more intensive field observations afterwards. It is anticipated that much of the work on Rarotonga will be carried out by Dr. Duane Gubler who will be working with the Pacific Research Section, beginning about August 1, 1971, as a guest worker.

Honors and Awards:

Dr. Rosen was appointed chairman of the Program Committee for the 1971 meeting of the American Society of Tropical Medicine and Hygiene. He continued to serve as Councilor of the same Society; as a member of the Editorial Board of its journal until the end of December, 1970; and as Clinical Professor of Tropical Medicine and Medical Microbiology at the University of Hawaii School of Medicine. He also chaired a symposium on "Intestinal Capillariasis of the Philippines" at the Fifth Regional Seminar on Tropical Medicine and Public Health held in Taipei, Taiwan under the sponsorship of the SEAMEO Central Coordinating Board for Tropical Medicine and Public Health.

Publications:

Ash, L.R.: Diagnostic morphology of the third-stage larvae of Angiostrongylus cantonensis, Angiostrongylus vasorum, Aelurostrongylus abstrusus and Anafilaroides rostratus (Nematoda: Metastrongyloidea). J. Parasit., 56:249-253, 1970.

Bunnag, T., Comer, D.H., and Punyagupta, S.: Eosinophilic myeloencephalitis caused by Gnathostoma spinigerum. Neuropathology of nine cases. J. Neurol. Sci., 10:419-434, 1970.

Punyagupta, S., Bunnag, T., Juttijudata, P., and Rosen, L.: Eosinophilic meningitis in Thailand. 2. Epidemiologic studies of 484 typical cases and the etiologic role of Angiostrongylus cantonensis. Amer. J. Trop. Med. Hyg., 19:950-958, 1970.

Char, D.F.B., and Rosen, L.: Eosinophilic meningitis. In Gellis, S.S., and Kagan, B.M. (eds.): Current Pediatric Therapy - 5. Philadelphia, W.B. Saunders, Co., 1971 (in press).

Rosen, L.: Angiostrongyliasis. In Hubbert, W.T., McCulloch, W.F., and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man - 6th Edition, (in press).

Serial No. NIAID 67G

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Role of enterotoxin-producing coliform bacteria in pediatric diarrhea.

Previous Serial Number: None

Principal Investigator: Dr. Andrew G. Dean

Other Investigators: Drs. Louis Harden, Yi-Chuan Ching, Ronald Williams, and Kay A. Kyser

Cooperating Units: Department of Pediatrics, U.S. Army Tripler General Hospital, Honolulu, Hawaii; Department of Pediatrics, Kaiser Medical Center, Honolulu, Hawaii; Pediatric Clinic, Schofield Barracks, Hawaii; U.S. Army Medical Research Unit, Kuala Lumpur, Malaysia.

Man Years:	NIAID staff
Total:	7/12
Professional:	7/12
Others:	

Project Description:

Objectives

To investigate the relationship between enterotoxin-producing coliform bacteria and pediatric diarrheal disease. To develop a more economical and more sensitive test for enterotoxin production than the rabbit ileal loop.

Methods Employed

Rectal swabs are collected from children with and without acute diarrheal disease in three outpatient clinics on Oahu and in a hospital for aborigines near Kuala Lumpur. In addition to routine culture, ten strains of coliform organisms from each specimen are tested for enterotoxin production in rabbit ileal loop segments or with a newly developed infant mouse system. Positive strains cause fluid accumulation and dilatation of the intestine.

In attempts to develop alternative biological tests, small intestinal loops of mice, rats, and baby chicks have been inoculated with a known positive strain of E. coli, and a variety of invertebrate animals have been immersed in water containing enterotoxin. Infant mice and rabbits have been inoculated intragastrically with toxin strains.

Major Findings

To date, from approximately a dozen specimens only one organism dilating rabbit loops has been isolated. The work has been hindered by inconsistencies in the reaction of individual rabbits and of different parts of each rabbit's intestine to known positive control strains. The need for a less cumbersome and more reliable test has become obvious.

Although the other alternate systems tried either did not respond to enterotoxin or offered no advantage over the adult rabbit ileal loop, direct inoculation of the stomach of infant mice and observation of the small intestine several hours later has so far proved to be a reliable and convenient test for enterotoxin. Experiments are underway to further compare this test with the rabbit ileal loop, using a number of known positive and negative strains of E. coli.

Significance to Biomedical Research and the Program of the Institute

At present detection of enteropathogenic E. coli depends on recognition of certain serotypes known to have been associated with diarrheal disease in the past. These serotypes are found occasionally in normal individuals however, and not all strains of a given serotype necessarily have the same pathogenicity. Tests for enterotoxin production, by measuring the apparent pathogenetic factor, offer hope of establishing a more direct relationship between a given coliform organism and its pathogenic potential. Epidemiologic surveys such as the present study are necessary to establish a relationship between laboratory tests for toxin production and actual causation of disease in the human population.

The need for better tests of enterotoxin production is paramount. The infant mouse test, for example, reduces the necessary time and expense by several fold, and should greatly facilitate epidemiologic surveys.

Proposed Course of the Project

Evaluation and improvement of the infant mouse test for enterotoxin will be continued, and it will be used to complete the survey of pediatric diarrhea. It is planned to terminate the project temporarily in mid-August, 1971, when the principal investigator leaves for nine months of academic work.

Honors and Awards: None

Publications: None

LABORATORY OF IMMUNOLOGY

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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
July 1, 1970 through June 30, 1971

The Laboratory of Immunology has enjoyed a year of considerable scientific progress and productivity. This has been achieved despite the fact that three scientists of international stature left the Laboratory to assume posts in other institutions. Dr. Baruj Benacerraf, Chief of the Laboratory from 1968 through 1970, resigned in order to accept the Fabyan Professorship of Comparative Pathology at Harvard Medical School and to become Chairman of the Department of Pathology at that institution. Dr. Ralph Reisfeld left the Laboratory to become a Full Member of the Division of Experimental Pathology at the Scripps Clinic and Research Foundation and Dr. Carl Pierce accepted the position of Assistant Professor of Pathology at Harvard Medical School.

Dr. William Paul was appointed Acting Chief of the Laboratory in July, 1970 and Chief of the Laboratory in January 1971. Two associates, Dr. Charles Janeway, Jr. and Dr. John Stobo, joined the Laboratory during the past year. In addition, Dr. Alberto Chersi, Dr. John van Boxel and Dr. Raphael Sharon entered the Laboratory through the Visiting Program and Dr. Latham Claflin has held a post-doctoral fellowship at the Laboratory. A series of guest workers, fellows and visiting scientists completed their tenure in the Laboratory during the past year. They were Dr. Thomas Francis, Dr. Yehudit Stupp, Dr. Harriet Gershon, Dr. John Martin, Dr. Ken Gray, Dr. Jack Pincus, Dr. Rene Rouques, Dr. Margaret Goldman and Dr. Helena Selawry. Most of these scientists have made significant research contributions during their stay in the Laboratory.

The members of the Laboratory have played an important role in the immunologic community. Dr. Mage and Dr. Paul served on the Program Committee for the annual meeting of the American Association of Immunologists and Dr. Green and Dr. Paul chaired sessions at that meeting. Dr. Mage was invited to lecture at the New York Academy of Sciences Meeting on Immunoglobulins and to both lecture at a symposium and co-chair a workshop at the First International Congress of Immunology. Dr. Green was asked to co-chair a workshop at the International Congress and Dr. Paul delivered the Summary Address at the Second International Convocation on Immunology, presented one of the lectures in the series Recent Progress in Immunology, at NIH, and has been invited to speak at a symposium at the International Congress of Immunology.

The Laboratory of Immunology has as its basic goal the exploration of fundamental mechanisms in cellular immunology, immunogenetics, immunochemistry, immunopathology and tumor immunology. To these ends, studies of cellular interactions in the immune response, mechanisms of induction of specific immune tolerance, nature of antigen binding receptors of lymphocytes and genetic control of specific immune responses have been actively pursued. Mechanisms of cell mediated cytotoxicity, activation of the complement cascade and immunity to a transplantable leukemia are being studied. Further, the genetic control of immunoglobulin synthesis and variability as well as the control of activation of immunocompetent cells are areas of active investigation.

Antigen Binding Receptors of Lymphocytes

Lymphocytes mediating specific immune responses have been postulated to be activated as a result of the interaction of antigen with cell surface receptors. Studies by Dr. Joseph Davie and Dr. William Paul of the Laboratory of Immunology have demonstrated the existence of cell surface receptors on cells mediating cellular immune responses by the fact that these cells specifically adhere to antigen coated agarose beads. Using such beads to specifically remove cells from a population immune to a hapten-carrier conjugate, the receptors of these cells have been shown to have a complex specificity pattern involving elements of hapten and carrier. On the other hand, another group of cells has been demonstrated, through radioautographic and immunofluorescent experiments, to have surface immunoglobulin molecules which act as receptors and which are highly hapten specific in their binding of antigen. These studies point to the existence of two subsets of lymphocytes bearing receptors with qualitatively different specificity and, very likely, having very different numbers of these receptors.

Cellular Interactions in the Immune Response

Antibody responses to a variety of antigens involve the interaction of two specific types of lymphocytes. One type is the precursor of antibody-forming cells (AFCP) while the other exerts an important, but undefined, function in the activation of AFCP and is consequently referred to as a helper cell. Studies in this Laboratory, by Drs. Katz and Paul, in collaboration with Dr. B. Benacerraf, have markedly aided in the elucidation of this system and have specifically demonstrated a difference in the effective specificity of these cell types, the radiation resistance of activated helper cells, and the capacity of an active graft versus host response to obviate the need for helper cells. Additional studies are currently in progress to identify the precise mechanism of action of helper cells.

Activation of Murine Lymphocytes and Thymocytes by Conconavalin A

A series of agents exist which specifically bind to cell surfaces. Some among these cause division and transformation by lymphocytes. Concanavalin A (Con A), a protein obtained from the jack bean, is one such agent which binds to a variety of cells, including tumor cells and lymphocytes. Drs. Stobo and Paul have shown that Con A causes DNA synthesis on the part of both lymphocytes and thymocytes but not of bone marrow lymphocytes, although it binds equally well to each of these cell types. Thus, bone marrow, thymus and spleen cells can bind approximately 10 million Con A molecules per cell with an effective equilibrium constant of $\sim$ 1x10^6 L/M. Electron micrographs prepared by Dr. A. Rosenthal of LCI using conjugates of Con A and horse radish peroxidase and of Con A and ferritin demonstrate a patchy distribution of surface binding. Activation of thymus and spleen cells is optimal when approx mately 25% of cell receptors have been occupied by Con A. Finally, responsive ness to Con A appears to be a property of relatively mature thymus cells as cells obtained from newborn and young animals demonstrate a poor response, although Con A is bound by these cells. These studies have demonstrated that activation of lymphoid cells by mitogens requires a cell surface interaction but that the binding of the agent is not necessarily sufficient to trigger the cell.

Specificity of Activation of Immunocompetent Cells

The antibody response of animals to a given antigenic stimulus has been traditionally considered to be a highly specific phenomenon. Nonetheless, it is well known that early in the immune response, a considerable amount of immunoglobulin is produced which cannot be demonstrated to be specific for the immunizing antigen. Through the use of a panel of hapten derivatized erythrocytes for enumeration of antibody forming cells by specific hemolysis in gel, Drs. Merchant and Inman have demonstrated that concomitant with the synthesis of antibodies specific for the immunizing hapten, antibody more specific for structurally related hapten is also produced. Indeed, many antibody forming cells secrete molecules which fail to lyse erythrocytes coated with the immunizing hapten but which lyse cells coated with an alternate, related compound. This finding is especially prominent early in the course of the immune response and, in general, is more marked among IgM producing cells than among IgG producing cells. Indeed, in the population of IgM synthesizing cells, there are more cells secreting antibody more reactive with one or another related hapten than there are cells producing antibody highly specific for the immunizing hapten. These studies may lead to insight into the specific activation mechanism of immunocompetent cells.

Linkage of Histocompatibility and Immune Response Genes

Immune responses to certain simple synthetic antigens have been demonstrated to be dependent on a single autosomal dominant gene. In the guinea pig, the PLL gene controls the response to poly-L-lysine and haptenic derivatives of this and several related polymers. Furthermore, the immune response to very small doses of bovine serum albumin (BSA) is also controlled by the PLL gene. Studies by Drs. Ellman and Green of LI, in association with Dr. Benacerraf of Harvard Medical School, during the past two years have demonstrated a very close linkage between the PLL gene and the major strain 2 histocompatibility genetic locus of guinea pigs. It has been possible to demonstrate this linkage both in offspring of matings of $(2x13)F_1$ and 13 guinea pigs and in the random bred Hartley and NIH populations. Furthermore, the demonstration of the existence of strain 2 histocompatibility antigens in outbred guinea pigs had led to a considerable delineation of the genetics of guinea pigs histocompatibility antigens.

Rose Lieberman of LI has recently delineated a new genetically controlled immune response in mice which is closely linked to histocompatibility genes. Thus, the antiidiotype and antiallotype antibody response of inbred mice to mouse IgA myeloma proteins requires that the responding strain possess different allotypic markers than the administered protein. Nonetheless, among strains with different allotypes from the antigen, a major difference in responsiveness exists and this difference is related to histocompatibility type. Mice of the $H2^a$ and $H2^k$ as well as $H2^s$ genotypes are responders whereas mice with other genotypes mount poor responses. The linkage to histocompatibility antigens has been formally demonstrated with the use of congenic resistant strains.

The linkage between immune responsiveness and histocompatibility type has major implications in pathogenesis of and susceptibility to disease.

Chemical Studies of Rabbit Immunoglobulin

Rabbit immunoglobulins are unique among those immunoglobulins thus far studied in that stable genetic markers (allotypes) exist in both the variable (V) and constant (C) regions of the heavy (H) polypeptide chain. A detailed analysis of the structural basis for and genetic linkage of these markers may provide key information in the determination of the genetic basis of antibody diversity. Furthermore, the occasional production of homogeneous antibodies by these animals may allow determination of the chemical basis of antibody specificity. Dr. Mage and Mrs. Young-Cooper have demonstrated that the genes controlling V region allotypic markers are closely linked to C region genes. However, one recombinant involving an a locus (V region) marker and markers of the C region of IgG H chains (A14-A15) has been observed in 216 trials demonstrating that the loci for these two regions of the IgG chain are separated. Furthermore, on the basis of detailed amino acid sequence analysis by Dr. Chersi and Dr. Mage, in collaboration with Dr. Appella of NCI, the chemical basis of the A14-A15 allotypic marker has been found to be a threonine-alanine interchange at position 309.

Guinea Pigs Deficient in the Fourth Component of Complement: The delineation of an early component bypass in the activation of complement

The activation of the complement cascade leads to the production of a series of substances causing immunopathologic reactions. A group of guinea pigs was discovered by Drs. Ellman and Green of LI and Dr. Frank of LCI last year which have a total deficiency of one of the early complement components, C4. A colony of these animals has now been established. They represent the only available animals with a total deficit of one of the early complement components. These animals, nonetheless, display normal Arthus reactivity which is dependent upon a leukocyte chemotactic agent released on the activation of late complement components and, in addition, late components of complement can be fixed from the serum of these animals by antigen-antibody complexes or by endotoxin. These findings demonstrate the existence of an alternate, and significant, pathway for the activation of late complement components. The C4 deficient guinea pigs represent a valuable resource in the delineation of immunopathologic phenomena mediated by the complement cascade and of the mechanisms of activation of various complement components.

Antibody Dependent Cell Mediated Cytotoxicity; the role of bone marrow derived lymphocytes

In vitro cell mediated cytotoxicity has been utilized as a model of both transplant and tumor immunity. Two general types of responses have been observed. In one instance, lymphoid cells from specifically sensitized animals kill target cells to which they are immune. This response has been shown by others to require the participation of thymus derived or thymus dependent lymphocytes (T lymphocytes). The other response involves the lysis of antibody coated target cells (generally erythrocytes) through the action of lymphoid cells from normal, non-sensitized animals. Work by Drs. van Boxel, Stobo, Green and Paul has now demonstrated that T lymphocytes are not required for this latter type of lysis but that bone marrow derived lymphocytes (B lymphocytes) play a major role in this response. Thus, spleen cells from thymus deprived animals and spleen cells from which T lymphocytes have been removed through the action of antisera directed at an isoantigenic marker of thymus and thymus derived cells, are fully competent to destroy antibody coated erythrocytes. On the other hand, specific removal of B lymphocytes through the use of anti-immunoglobulin serum and complement markedly depresses the capacity of a population of spleen cells to mediate lysis. These results point to a potential role for the cooperative effect of antibody and B lymphocytes in the mediation of a variety of immunopathologic phenomena.

Serial No. NIAID - 140
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Receptors on Immunocompetent Cells

Previous Serial No: None

Principal Investigator: Dr. W. E. Paul

Other Investigators: Project Subtitle I: Dr. J. M. Davie
Dr. A. S. Rosenthal, LCI, NIAID
Project Subtitle II: Dr. J. M. Davie
Dr. R. G. Mage
Dr. M. B. Goldman

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-7/12			4-7/12
Professional	2-1/12			2-1/12
Other	2-6/12			2-6/12

Project Subtitle I: Antigen-Binding Receptors on Guinea Pig Lymphocytes.

Project Description:

Objectives:

To evaluate the specificity and character of the antigen specific receptors on various classes of immunocompetent cells.

Methods Employed:

Direct binding of radiolabeled antigen to the surface of lymphoid cells was evaluated by autoradiography. Antigen binding cells were specifically removed from cell populations by adsorption to antigen-agarose bead conjugates

Major Findings:

Previous studies using guinea pigs immunized to dinitrophenylated guine pig albumin (DNP-GPA) demonstrated a difference between the receptors of cells mediating a cellular immune function (i.e., antigen-mediated stimulation of DNA synthesis) and the receptors of cells secreting antibody. Thus, "cellular immune" cells would adsorb to agarose beads to which DNP-GPA had been covalently linked but would not adsorb to beads coated with DNP conjugated to other carriers. On the other hand, antibody secreting cells would adsorb to agarose beads coated with DNP compounds irrespective of the carrier. These studies suggested a difference in the specificity of the receptors, but did not rule out other reasons for the apparent difference.

Methods allowing direct evaluation of antigen binding were developed to measure receptor specificity more precisely. When lymphoid cells from non-immune guinea pigs were examined, rare lymphocytes were found which bound sufficient ^{125}I-DNP-GPA to their surfaces to be detected by autoradiography. These cells were found in lymph node, spleen, peripheral blood and bone marrow, but not in thymus, and occurred with a frequency of about 40 per 100,000 lymphocytes. An examination of fetal guinea pigs demonstrated that ^{125}I-DNP-GPA antigen binding cells (ABC) appeared in the spleen between 40 and 50 days of gestation. This finding indicates that these cells probably do not arise because of prior antigenic contact.

The receptors of DNP-GPA ABC appear to be largely hapten specific in that: 1) free ϵ-DNP-L-lysine inhibits binding of ^{125}I-DNP-GPA, 2) DNP-bovine serum albumin (DNP-BSA) and DNP-GPA are equivalent in their capacity to inhibit the binding of ^{125}I-DNP-GPA to cells, and 3) the DNP-GPA ABC adsorb equally well to DNP-BSA agarose and DNP-GPA agarose. Anti-immunoglobulin, especially anti-γ_2 antisera, block the binding of ^{125}I-DNP-GPA to ABC indicating that the receptors of most DNP-GPA ABC are immunoglobulin of the γ_2 class.

When guinea pigs are immunized with 50 μg DNP-GPA emulsified in complete Freund's adjuvant, a striking increase in the frequency of DNP-GPA ABC occurs. This increase was shown not to be related to the adsorption of cytophilic antibody to lymphocyte surfaces. The most rapid increase in ABC occurs prior to the appearance of detectable serum anti-DNP antibody or of anti-DNP antibody secreting cells. The receptor characteristics of the ABC of immune guinea pigs are similar to those found previously for ABC's of non-immune guinea pigs. The receptors were shown to be highly hapten specific and appeared to be γ_2 immunoglobulin. One major difference between the receptors of DNP-GPA ABC of non-immune guinea pigs and those of DNP-GPA ABC from immune guinea pigs was the marked increase in susceptibility of the latter to inhibition of ^{125}I-DNP-GPA binding by DNP-lysine. For example, 50% of DNP-GPA ABC from non-immune guinea pigs could be inhibited from binding ^{125}I-DNP-GPA by 10^{-3} to 10^{-4} M DNP-lysine, whereas 30 days after immunization the concentration required for similar inhibition had dropped to 10^{-8} to 10^{-9} M DNP lysine. This remarkable change appears to reflect an antigen driven selection of ABC's having high receptor affinity for DNP-lysine. It is well known that serum antibody undergoes a similar change in affinity in the course of immune responses.

The characteristics of DNP-GPA ABC of both nonimmune and immune guinea pigs resemble circulating antibody molecules in that they are immunoglobulin, they are hapten-specific, and an avidity increase occurs in the course of the immune response. Therefore, ABC have receptors consistent with those postulated for precursors of antibody forming cells. These characteristics are entirely different from those of cells which mediate cellular immunity. The receptors of these latter cells have a high degree of carrier specificity.

While we have not evaluated the receptors of "cellular immune" precursors lymphocytes in non-immune guinea pigs it is reasonable to assume that they too will have carrier specificity. The guinea pig, therefore, appears to have two separate classes of immunocompetent cells with receptors of differing specificity.

Research is currently underway attempting to demonstrate directly the differentiation of antigen binding cells (? antibody-forming cell precursors) to mature antibody secreting cells. Preliminary results demonstrate similar changes in avidity of the receptors of antigen binding cells and the antibody secreted by plaque forming cells.

Significance to Biomedical Research and the Program of the Institute:

The mechanism by which antigens are recognized by cells is a basic question of immunology. The demonstration of two distinct recognition systems in the guinea pig, one possessed by "cellular immune" cells, the other by antibody forming cells and their precursors, is evidence that these two classes of immunocompetent cells have separate origins.

Proposed Course of Project:

Attempts will be made to define the selectional pressures and mechanisms leading to the "maturation" of an immune response; that is, the presumed antigen driven events which favor the proliferation of cells with high affinity receptors over those with low affinity receptors. In addition, further work on the nature of the receptors of cellular immune cells (or the thymus derived lymphocyte) will be carried out and attempts to obtain membrane fragments and soluble materials with receptor activity will be made.

Project Subtitle II: Membrane Associated Immunoglobulin on Rabbit Lymphocytes

Project Description:

Objectives:

To determine whether lymphocytes of rabbits genetically capable of producing two allelic forms of light (L) chains have one of both forms on their surface.

Methods Employed:

Antibodies specific for each of two allelic forms of rabbit L chains, b4 and b5, were labeled with fluorescein or radioiodine. Direct binding of the antibodies to the surface of lymphocytes was evaluated by immunofluorescence and/or autoradiography.

Major Findings:

It is well known that individual plasma cells of rabbits genetically capable of producing two allelic forms of light chains are phenotypically restricted to the synthesis of one of these L chain types. This phenomenon is referred to as alleleic exclusion. The recent demonstration of immunoglobulin on the surface of lymphocytes which were not secreting antibody offered an opportunity to determine whether or not lymphocytes not yet differentiated to the stage of antibody secretion were restricted phenotypically. As such cells represent, at least in part, a precursor cell pool, this information would be important in an understanding of the events involved in specific cellular differentiation and antigen mediated activation of precursor cells.

A method was devised to directly evaluate individual lymphocytes for the presence of either or both alleleic forms of light chains. Antibodies specific for the b4 or b5 allele were purified and cross absorbed. The antibodies were then radio-iodinated or fluoresceinated. By staining lymphocytes in sequence with the reagents and then evaluating single lymphocytes for silver grains and for fluorescence, the distribution of light chains could be determined. It was found that most lymphocytes of heterozygous rabbits expressed only one of the two alleles. No more than 3% of lymphocytes had both forms. Therefore, allelic exclusion holds for most small lymphocytes as well as for antibody secreting cells.

Significant to Biomedical Research and the Program of the Institute:

Since all cells presumably have the genetic information to make all immunoglobulins, the factors operative in limiting the production by any single cell to a limited number of antibodies are crucial to our understanding of the clonal theory of immunity. The extreme restriction which we have demonstrated here makes it likely that antibodies of a single specificity are found on most lymphocytes.

Proposed Course of Project:

The small, definite, number of lymphocytes which produce both forms of immunoglobulin suggest that a pool of less restricted, possibly less mature, cells exists. We propose to study these cells by searching for the organ of origin of these cells, determining the factors which affect their frequency, and determining their ultrastructure and evaluating their function.

Publications:

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Paul, W.E. and Kask, A.M. Structural control of immunogenicity. I. Synthesis of oligo-L-lysine peptides and their mono-ϵ-DNP derivatives. Immunology, in press.

Stupp, Y., Paul, W.E. and Benacerraf, B. Structural control of immunogenicity. II. Antibody synthesis and cellular immunity in response to immunization with mono-ϵ-DNP-oligo-L-lysines. Immunology in press.

Stupp, Y., Paul, W.E. and Benacerraf, B. Structural control of immunogenicity. III. Preparation for and elicitation of anamnestic antibody responses by oligo- and poly- lysines and their DNP derivatives. Immunology, in press.

Paul, W.E., Stupp, Y., Siskind, G.W. and Benacerraf, B. Structural control of immunogenicity. IV. Relative specificity of elicitation of cellular immune responses and of ligand binding to anti-hapten antibody after immunization with mono-ϵ-DNP-nona-L-lysine. Immunology, in press.

Paul, W.E. and Davie, J.M. Antigen binding receptors on lymphoid cells. Nature and specificity of receptors on various types of immunocompetent cells. Progress in Immunology, in press.

Serial No. NIAID - 141
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Specific Cellular Interactions in Immune Responses to Hapten-Carrier Conjugates

Previous Serial No: 146

Principal Investigator: Dr. W. E. Paul

Other Investigators:	Project Subtitle I:	Dr. David H. Katz Dr. J. M. Davie
	Project Subtitle II:	Dr. John Stobo
Cooperating Units:	Project Subtitle I:	Dr. Baruj Benacerraf Harvard Medical School
	Project Subtitle II:	Dr. R. K. Gershon Yale University

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-5/12			4-5/12
Professional	1-11/12			1-11/12
Other	2- 6/12			2-6/12

Project Description:

Project Subtitle I: Cellular Interactions in Guinea Pigs and Rabbits

Objectives:

Immune responses involving the stimulation of antibody synthesis have been shown to either require or be enhanced by the cooperative interaction of different sets of specific lymphocytes. The mechanism underlying this interaction and the nature of the lymphocyte classes involved have been investigated in detail utilizing inbred guinea pigs and hapten-carrier conjugates as antigens. These studies were undertaken in order to gain insight into the regulatory mechanisms of immune cell activation and to delineate techniques which might specifically augment or suppress immune reactions.

Methods Employed:

Inbred guinea pigs were primed with 2,4-dinitrophenyl (DNP)-ovalbumin (OVA) in saline and, at various times thereafter, received either a supplemental immunization with a different protein, such as bovine gamma globulin (BGG), or an infusion of syngeneic or allogeneic lymphoid cells. They were subsequently challenged, usually with DNP-BGG, and serum anti-DNP antibody concentrations measured at various times by an antigen binding assay. Additionally, antibody responses, both in vivo and in vitro using a Mishell-Dutton culture system, were evaluated by specific hemolysis in gel by plaque forming cells.

Major Findings:

1. Guinea pigs and rabbits which are primed with DNP-OVA make little or no secondary anti-DNP response upon challenge with DNP-BGG. This phenomenon has been termed carrier specificity. However, if they receive an immunization with BGG in complete Freund's adjuvant (CFA) after primary immunization with DNP-OVA and before secondary challenge with DNP-BGG, the latter challenge leads to a marked synthesis of anti-DNP antibodies. This effect is mediated by carrier-specific lymphocytes as shown by the ability of lymph node cells from inbred guinea pigs immunized to BGG to transfer to syngeneic DNP-OVA primed recipients the capacity to respond to DNP-BGG. This experiment indicates the participation of carrier specific "helper" cells in the activation of precursors of antibody forming cells.

2. The capacity of "helper" lymphoid cells from BGG immunized animals to enhance the anti-DNP antibody response to DNP-BGG of syngeneic DNP-OVA primed recipients is not abolished by in vitro irradiation of the cells. Doses of x-ray of up to 5000 R administered to BGG-specific lymphoid cells do not diminish the magnitude of the anti-DNP response to DNP-BGG of recipients of these cells.

3. Primary in vitro antibody responses specific for the trinitrophenyl (TNP) group can be augmented by the presence of carrier specific helper cells. Thus, spleen cells from BALB/c mice primed 3 days earlier with unconjugated burro erythrocytes, yielded a considerably greater in vitro anti-TNP response to TNP-burro erythrocytes than did normal spleen cells. Similarly, spleen cells from mice primed with the bacteriophage Φx 174 show enhanced anti-TNP responses to TNP-Φx 174. The establishment of an in vitro model for cell cooperation promises to speed the elucidation of the mechanism of this phenomenon.

4. Rabbits primarily immunized with type III pneumococci and then supplementally immunized with BGG display greatly enhanced in vivo secondary anti-S_{III} antibody responses when challenged with an S_{III}-BGG complex whereas only small secondary responses to S_{III}-BGG are observed in rabbits not given a supplemental BGG immunization. This study demonstrates the potential practical applications of such a system whereby heightened antibody responses to a variety of bacterial polysaccharides may be feasible.

5. The transfer of donor lymphoid cells from inbred guinea pigs to allogeneic recipients previously primed with DNP-OVA results in stimulation of anti-DNP and anti-OVA antibody synthesis in the absence of any further antigenic challenge of the recipients. Furthermore, when such recipients of allogeneic cells are challenged with DNP-BGG 6 days after transfer, they display significantly enhanced secondary anti-DNP antibody responses regardless of whether or not the allogeneic cells employed came from BGG-immunized donors. The phenomenon results from the induction of a graft-versus-host reaction in the recipient as evidenced by the failure to elicit the effect when irradiated strain 2 guinea pig donor cells, L_2C leukemia (a strain 2 leukemia) donor cells, or $(2x13)F_1$ hybrid donor cells are transferred to strain 13 recipients. The concomitant occurrence of a host-versus-graft response is not required for the elicitation of this phenomenon as F_1 hybrid recipients of parental donor cells exhibit this effect. The graft-versus-host reaction appears to make possible the direct stimulation of hapten-specific antibody forming cell precursors as evidenced by the stimulation of marked secondary anti-DNP responses in recipients of allogeneic cells following challenge with a non-immunogenic DNP-copolymer, DNP-poly D glutamic acid-D-lysine (DNP-D-GL). Thus, the graft-versus-host reaction appears to obviate the need for carrier-specificity in the development of anamnestic anti-hapten antibody responses.

6. Injection of DNP-D-GL leads to a state of profound DNP-specific immunologic tolerance. This is evidenced by the inability to elicit subsequent primary anti-DNP antibody responses to DNP-OVA. The tolerance appears to be central in origin as evidenced by diminished frequency both of DNP-specific antibody-forming cell precursors and of DNP-specific antibody-secreting cells. Furthermore, a similar state of tolerance can be achieved in animals previously primed with DNP-OVA by administration of DNP-D-GL prior to secondary challenge with DNP-OVA. This state of hapten-specific tolerance appears to reflect a specific suppression of antibody forming cell precursors.

Significance to Biomedical Research and the Program of the Institute:

Normal immune responses to a wide variety of naturally occurring antigens appear to involve the participation of both "helper" cells and antibody-forming cell precursors. The manipulation of both of these cells should allow specific enhancement or suppression of immune responses. The latter has been achieved both for artificial antigens and for a polysaccharide of a pneumococcus of considerable medical importance. Similarly, hapten-specific tolerance has been achieved and the similar approach of integrating given determinants into non-immunogens may lead to medically important tolerance, as for example with fragments of transplantation antigens and non-immunogenic carriers.

Proposed Course of Project:

This problem will be pursued in a variety of ways, both on a fundamental and applied level. First, efforts to determine the precise function of helper cells will be made utilizing *in vitro* systems. Second, a more detailed evaluation of the mechanism of tolerance induction by non-immunogens will be undertaken

Third, the SIII-protein conjugates, as well as similar conjugates of other weakly immunogenic bacterial polysaccharides, will be studied in detail as potential vaccines.

Project Subtitle II: Activation of Immunocompetent Cells of Mice.

Project Description:

Objectives:

The mechanisms of activation of thymus derived (T) lymphocytes and the contribution of these cells to the activation of bone marrow derived (B) lymphocytes were studied in mice because the availability of cell markers and the capacity to specifically ablate the T cell component makes this species particularly useful for detailed evaluation of cellular activation and interaction.

Methods Employed:

Inbred mice were thymectomized as adults, lethally irradiated one week later and reconstituted with syngeneic bone marrow cells. These mice and normal animals were used as donors of spleen cells for in vitro studies of activation by either conconavalin A (Con A) or phytohemagglutinin (PHA) as measured by stimulation of DNA synthesis. Further, measurements of the numbers of T and B lymphocytes in these populations were made in quantitative cytotoxicity tests with anti θ isoantisera and anti-immunoglobulin antisera. Thymus deprived mice, and similar mice reconstituted with thymocytes, were immunized with either 2,4-dinitrophenyl-keyhole limpit hemocyanin (DNP-KLH) or DNP-bovine serum albumin (DNP-BSA). Serum ϵ-DNP-L-lysine binding capacities were measured and affinities were determined by a relationship derived in the course of this study. That relation is:

$$K = \frac{[(B/F)_x \cdot B_i \cdot R] - [(B/F)_i \cdot B_x]}{(B_i \cdot B_x)(1-R)}$$

where K is equilibrium constant, $(B/F)_i$ is the ratio of bound to free ligand at the index condition; B_i is the bound ligand concentration at the index condition; $(B/F)_x$ is the ratio of bound to free ligand at the test condition; B_x is the bound ligand concentration at the test condition, and R is the ratio of antigen binding capacity at the test condition to antigen binding capacity at the index condition

Major Findings:

1. Con A is slightly superior to PHA in the stimulation, in vitro, of DNA synthesis by spleen cells obtained from normal mice. On the other hand, thymus cells respond very poorly to PHA but mount an excellent response to Con A. Bone marrow cells respond neither to Con A nor to PHA.

2. ^{125}I-Con A binds to the surface of spleen cells, bone marrow cells and thymus cells. By analyzing binding data by a Scatchard plot, it was shown that these cells can bind, on the average, 10^7 molecules of Con A per cell with an effective equilibrium constant of 10^6 L/M. No difference was observed between cells which could be stimulated with Con A and those which could not be stimulated. Furthermore, spleen cells could be separated into a population which bound relatively large amounts of Con A and one which bound relatively small amounts of Con A on the basis of adsorption to and elution from Con A-polyacrylamide columns. Despite the difference in the degree of binding of these cell populations, no appreciable difference in Con A stimulated DNA synthesis was observed.

3. Spleen cells from thymus deprived mice are not stimulated to synthesize DNA by PHA when they are cultured at 1×10^6 cells per tube but they retain a significant degree of responsiveness to Con A. This response to Con A can be abolished by pretreating the cell population with anti θ and complement (C) but not by treating with anti-immunoglobulin and C. This suggests that subpopulations of thymus derived cells exist and that these populations differ in their responsiveness to these mitogens. Preliminary evidence indicates that cooperation between these populations is involved in lymphocyte activation.

4. Thymus deprived mice produce less anti-DNP antibody after immunization with either DNP-BSA or DNP-KLH than do reconstituted animals. Furthermore, the affinity of the anti-DNP antibody produced by thymus deprived mice is lower than that produced by thymus cell reconstituted mice or sham thymectomized animals. This finding strongly implies an active role of T lymphocytes in the selective process which leads to emergence of high affinity antibody secreting cells.

Significance to Biomedical Research and the Program of the Institute:

These models of activation of the major subclasses of lymphoid cells in mice provide powerful tools for the precise delineation of immunocompetent cell activation. This should, in turn, lead to an understanding of methods of controlling the response in desired ways which has obvious usefulness in immunopathologic processes.

Proposed Course of Project:

A detailed analysis of cellular interaction in mitogen mediated stimulation will be undertaken as well as a search for cell types not bearing the typical B and T cell markers.

Publications:

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Katz, D.H., Bluestein, H.G., Rouques, R.J. and Pierce, C.W. Anti-TNP antibody responses in vitro: Augmented primary responses of carrier-primed spleen cells. J. Immunol. 106: 271, 1971.

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Serial No. NIAID - 142
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genetic Control of the Immune Response

Previous Serial No: 140

Principal Investigator: Dr. Ira Green

Other Investigators:

Project Subtitle I:	Dr. B. Benacerraf Harvard Medical School Boston, Mass.
Project Subtitle II:	Dr. L. Ellman Dr. B. Benacerraf
Project Subtitle III:	Dr. L. Ellman Dr. J.K. Inman
Project Subtitle IV :	Dr. L. Ellman Dr. M. Frank, LCI, NIAID
Project Subtitle V :	Dr. J. vanBoxel Dr. A. Steinberg, AR, NIAMD

Cooperating Units: Michael Frank, LCI
Dr. Alfred Steinberg, NIAMD

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-7/12			3-7/12
Professional	1-7/12			1-7/12
Other	2			2

Project Subtitle I: Genetic Control of Immune Responsiveness to Limiting Doses of Proteins and Hapten Proten Conjugates in Guinea Pigs

Project Description:

Objectives:

The ability of guinea pigs to respond to a simple synthetic poly and amino acid antigen, poly-L-lysine (PLL), is determined by the presence of an autosomal dominant gene (PLL gene). This gene is variably present in Hartley guinea pigs, present in all strain 2 guinea pigs and absent in strain 13 guinea pigs. Previous studies (Green, I., Inman, J.K. and Benacerraf, B. PNAS 66:1267, 1970) have demonstrated that this gene also determines the immune response of guinea pigs to limiting doses of the ordinary protein antigen, bovine serum albumin (BSA). That is strain 2, $(2x13)F_1$, and PLL gene (+) F_1 x 13 backcross animals could respond to limiting doses of BSA whereas strain 13 animals and PLL (-) F_1 x 13 backcross animals failed to respond. All Hartley PLL responder animals responded well to limiting doses of BSA. Hartley PLL non responder animals either failed to respond or made an anti BSA antibody response significantly lower than that of responder animals.

In the present study these experiments were extended to explore the relationship between the presence of the PLL gene and the immune response to limiting doses of other albumins, other proteins, and hapten protein conjugates.

Methods Employed:

Strain 2, strain 13, $(2x13)F_1$ x 13 backcross and Hartley guinea pigs were immunized with limiting doses of human serum albumin (HSA), ovalbumin (OVA) and 4 other unrelated protein antigens as well as various 2,4, dinitrophenyl conjugates of guinea pig albumin and BSA.

The anti-protein antibody response was measured by the Farr technique using ^{125}I labeled protein antigens; the anti-DNP antibody response was also measured by the Farr technique using ^3H-DNP-lysine. The responder status of the Hartley animals and the F_1 x 13 backcross animals was evaluated by the response to immunization with DNP-PLL. The backcross animals were also injected with the L_2C leukemia which readily kills only strain 2 and F_1 guinea pigs. This was done to evaluate the presence of the major strain 2 histocompatibility antigen in these animals.

Major Findings:

Only strain 2 and F_1 animals could respond to limiting doses of HSA. Strain 13 animals failed to make a response. The fact that strain 2 and strain 13 animals made an equivalent response to limiting doses of OVA and 4 other non albumin protein antigens indicates that the ability to respond to limiting doses of HSA and BSA in inbred guinea pigs is related in some way to a specific immune response gene.

Also, only those F_1 x 13 backcross animals capable of responding to limiting doses of BSA were PLL + and died as a result of an injection of the L_2C leukemia. Thus, this single experiment demonstrates both the linkage between an immune response gene controlling the response to PLL and the ability to respond to protein antigens, and the fact that these properties are in turn linked to the major strain 2 histocompatibility locus. The response of random bred Hartley animals to limiting doses of HSA did not demonstrate a clear relationship to the PLL gene. Thus, although the response to BSA appears linked to the PLL gene, the response of Hartley guinea pigs to the cross reactive HSA suggests that these immune response genes are distinct genetic factors.

The response of inbred guinea pigs to limiting doses of DNP-BSA conjugates was also revealing of role of the carrier molecule. Strain 2 guinea pigs immunized with 1 µg DNP_7BSA made an anti BSA and anti DNP antibody response. Strain 13 animals failed to make either responses. When strain 2 and 13 animals were immunized with 1 µg $DNP_{31}BSA$ neither strain made a significant anti DNP antibody response. The suggested explanation for these findings is that the low dose BSA carrier "determinant" is intact in the lightly derivitized DNP_7BSA, whereas the site in the molecule is destroyed or altered in the highly derivitized $DNP_{31}BSA$ molecule.

Significance to Biomedical Research and the Program of the Institute:

Genetic control of immune responses to simple synthetic poly amino acid antigens has been firmly established in both guinea pigs and mice. Furthermore, these "immune response genes" are linked in both species to the presence of major histocompatibility antigens. This study now demonstrates that the same or similar genetic factors (also linked to histocompatibility type) also control the immune response to certain complex protein antigens. Thus, the expectation that an understanding of the genetic control of immune response to simple synthetic antigens would lead to an insight into the response to complex protein antigens has been satisfied. These studies strongly suggest that the immune response to all antigens may be under genetic control. Finally, since the susceptibility to murine viral oncogenesis as well as the prevalence of certain human tumors, acute leukemia and Hodgkin's disease, are also linked to histocompatibility type, it may be that genetically controlled differences in the ability to mount an immune response against oncogenic viruses is important in relative resistance to development of various tumors.

Project Subtitle II: Relationship of Histocompatibility Type to Immune Response Genes

Project Description:

Objectives:

Previous studies from the Laboratory of Immunology have demonstrated a close linkage or identity of a specific immune response gene, the PLL gene, and the major histocompatibility locus of the inbred strain 2 guinea pig in both $(2x13)F_1$ x 13 backcross animals and random bred Hartley guinea pigs. The present investigation confirms and extends these earlier findings.

Methods Employed:

Anti-strain 2 isoantisera were produced in strain 13, Hartley PLL responder and Hartley PLL non-responder guinea pigs. The ability of these antisera to detect strain 2 histocompatibility antigen was investigated using the ^{51}Cr release cytotoxicity assay. The presence of strain 2 histocompatibility antigen was also studied by susceptibility of guinea pigs to the L_2C, leukemia, a strain 2 guinea pig leukemia.

In agreement with earlier findings, a new strain 13 anti-strain 2 antiserum was able to distinguish, without exception, between Hartley PLL responder and non-responder guinea pigs. Anti-strain 2 antiserum raised in Hartley responder guinea pigs, known to possess the strain 2 major histocompatibility antigen, was unable to distinguish between strain 2 and strain 13 lymphocytes, and between Hartley responder and non-responder lymphocytes. Conversely, anti-strain 2 antiserum raised in Hartley non-responder guinea pigs was readily able to distinguish between strain 2 and strain 13 lymphocytes and between Hartley responder and Hartley non-responder lymphocytes.

The strain 2 guinea pig leukemia was readily rejected by PLL non-responder $(2x13)F_1$ x 13 backcross animals while it was uniformly fatal for the PLL responder backcross animals. This demonstrates by an independent technique that there is a linkage between the major strain 2 histocompatibility antigen and the PLL gene. However, the leukemia was readily rejected by Hartley guinea pigs irrespective of their PLL status. Strain 2 cells must therefore possess antigens not present in Hartley guinea pigs which enable the rejection of strain 2 cells.

The incidence of PLL responders in the NIH multipurpose strain is very low. A single animal of 90 tested was found to be PLL positive. Its cells were found to possess the strain 2 major histocompatibility antigen while cells from the 14 non-responder NIH animals studied were lacking in this antigen.

Significance to Biomedical Research and the Program of the Institute:

These studies further demonstrate the persistent association between the PLL gene and the major histocompatibility specificity of the strain 2 guinea pig. Moreover, this association extends to random bred populations. Whether a single gene codes for both functions or whether two very closely linked genes are involved has yet to be resolved.

The findings in the PLL system in the guinea pig and the IR-1 system in the mouse together with the observation that specific immune response genes control the immune response to limiting doses of natural antigens in both mice and guinea pigs, suggest that histocompatibility type may be an important determinant of immune responsiveness. The observation that certain murine and human leukemias and lymphomas are associated with particular histocompatibility phenotypes suggests that the role of immune response genes in the pathogenesis of human disease may be an important and fruitful

Proposed Course of Project:

Other simple synthetic poly and amino acid antigens are being used as immunogens in guinea pigs. These studies are being performed in collaboration with Dr. Bluestein and Dr. Benacerraf. Here also other examples of genetic control of the immune response have been discovered and preliminary studies suggest that these immune responses are also linked to histocompatibility antigens.

Project Subtitle III: Immune Response to Hydralazine

Project Description:

Objectives:

Hydralazine is an important anti-hypertensive medication, the use of which is occasionally associated with development of anti-nuclear antibodies and of a syndrome resembling systemic lupus erythematosis. In an attempt to develop an animal model of this disease, guinea pigs of various strains were immunized with an emulsion of hydralazine and complete Freund's adjuvant. The guinea pigs remained healthy and no anti-nuclear antibodies could be detected. However, it was discovered that all strain 13 guinea pigs developed a delayed hypersensitivity reaction to hydralazine while all strain 2 animals failed to show such a response. This observation has been extensively studied in order to determine the mechanism of immune responsiveness to hydralazine.

Methods Employed:

Strain 2 and 13 and Hartley strain guinea pigs were immunized with varying doses of hydralazine in adjuvant. Delayed hypersensitivity was studied by intradermal injection of hydralazine with visual and morphologic observation of the skin reaction. The presence of anti-hydralazine antibodies was studied by passive cutaneous anaphylaxsis and a Farr assay utilizing hydralazinated ovalbumin an antigen. The genetics of the immune response to hydralazine was investigated by immunizing $(2x13)F_1$ and $(2x13)F_1$ x 2 backcross animals. Differences in the acetylation detoxification pathway in strain 2 and strain 13 animals was also investigated using the rate of excretion of sulfamethazine as an indicator system.

Major Findings:

Strain 13 guinea pigs appear to be "responders" to hydralazine; they uniformly have a delayed hypersensitivity reaction and in the vast majority of cases anti-hydralazine antibody can be detected by both PCA and the Farr assay. Strain 2 guinea pigs appear to be "non-responders" to hydralazine; they uniformly fail to develop a delayed hypersensitivity skin reaction, even when immunized with large amounts of adjuvant, and anti-hydralazine antibody is produced less frequently and in smaller amounts. With an occasional exception, Hartley guinea pigs appear to be "responders". The ability to respond to hydralazine appears to follow autosomal dominant inheritance. All (2x13)F_1 guinea pigs, and approximately one-half (2x13)F_1 x strain 2 backcross animals were responders. Using a strain 2 anti-strain 13 antiserum and the ^{51}Cr release cytotoxicity assay, no association between responsiveness to hydralazine and possession of the strain 13 major histocompatibility locus could be detected in the (2x13)F_1 x strain 2 backcross offspring. No difference in the acetylation of sulfa methazine was detected following intravenous injection and measurement of urinary metabolites.

Significance to Biomedical Research and the Program of the Institute:

The marked difference in immune response to hydralazine between strain 2 and strain 13 guinea pigs may be due to the operation of a "specific immune response gene". The immune response to several synthetic and naturally occurring antigens has been demonstrated to be under the control of such genes in the mouse, the guinea pig and the rabbit. The fact that the immune response to a pharmacologic agent appears to be under the control of an immune response gene in guinea pigs is of considerable interest.

Project Subtitle IV: C_4 Deficient Guinea Pig

Project Description:

Objectives:

A strain of guinea pigs with a genetically controlled total deficiency of the fourth component of complement has recently been developed in the Laboratory of Immunology. These guinea pigs represent the first example of a total deficiency of a complement component reacting early in the complement sequence (before C_3) and therefore represent an unique opportunity to investigate the role of complement in immunological phenomena.

Methods Employed:

The immune response of C_4 deficient (C4D) heterozygous and normal guinea pigs were compared by a variety of techniques in order to explore the contribution of C4 to various aspects of immune phenomena.

Major Findings:

Contact and delayed hypersensitivity reactions were normal in the C4D animals. Antibody production was depressed for certain antigens, especially when used in limiting amounts. Cellular exudative responses to a foreign body were normal as were both direct and reverse passive Arthus reactions. Passive cutaneous anaphylaxsis was normal in the C4D animals. Immune clearance by the reticuloendothelial system of autologous erythrocytes coated with specific rabbit antibody was depressed in the C4D animals, and could be normalized by the addition of normal guinea pig serum as a source of C_4.

Significance to Biomedical Research and the Program of the Institute:

The presence of normal Arthus reactions in C_4D animals was an unexpected finding since the Arthus reaction is known to require the participation of neutrophil chemotactic factors generated by activation of late complement components. The C_4D animals therefore appear to possess a mechanism or alternate pathway of complement activation has been suggested by several in vitro studies by other investigators. The normal Arthus reaction of C_4D animals appears to represent the first in vivo demonstration of an alternate pathway of complement activation. Studies are underway to try to define the mechanism of operation of the by-pass mechanisms. These animals thus represent an unique and important resource for further studies as to the mechanism of complement action.

Proposed Course of Project:

These animals, as well as cells and blood from these animals, are being used for a variety of in vivo and in vitro studies. The susceptibility of these animals to endotoxin and to challenge with living gram negative bacteria is under active investigation. Also being studied is the ability of serum and lymphoid cells from these animals to mediate various type cytotoxic reactions on target cells.

Project Subtitle V: Immunogenicity and Tolerogenicity of Nucleic Acids in Guinea Pigs

Project Description:

Objectives:

Purified nucleic acids are believed to be non-immunogenic and to behave as haptens; recently, however, both antibody formation and tolerance induction to these materials have been demonstrated in NZB and B/W mice. The responses of guinea pigs to nucleic acids have not hitherto been extensively studied; guinea pigs have the advantage that humoral and cellular immune responses can be readily explored simultaneously. The purpose of this study is to utilize this advantage in an effort to elucidate and possibly categorize the immune responses to this class of compound in guinea pigs. This may in turn lead to a better understanding of their role in disease states.

Methods Employed:

1) Inbred (Strain 2 and 13) and Hartley guinea pigs were immunized with the purified synthetic double-stranded RNA's, poly-inosinic-poly-cytidylic acid (Poly I-Poly C) and poly-adenylic-poly-uridylic (Poly A-Poly U), both with and without adjuvant and also complexed with methylated bovine serum albumin as carrier. Antibodies were measured by a sensitive

ammonium sulphage precipitation technique using ^{14}C-labeled Poly I-Poly C.

2) Delayed skin reactivity and *in vitro* lymphoid cell stimulation to nucleic acids with and without carrier were studied in the same animals.

3) Specific immunological tolerance was induced by cylophosphamide given 24 hrs. after antigen administration.

Major Findings:

The results demonstrated that purified nucleic acids elicit the production of small amounts of antibody in guinea pigs; by this criteria they behave as weak antigens. Not only were antibodies produced following immunization with both nucleic acids in adjuvant, without carrier methylated bovine serum albumin (MBSA), but preliminary data show that even adjuvant may be unnecessary for the production of a humoral response. Thus fatal system anaphylaxis could be induced in guinea pigs immunized with Poly I-Poly C in adjuvant without MBSA. Not only is this an *in vivo* demonstration of an antibody response to nucleic acid without carrier, but has important clinical implications which are discussed below. However, when complexed with MBSA the anti RNA antibody response was of much greater magnitude than the responses observed when the nucleic acid were injected alone or with adjuvant.

Delayed cutaneous skin reactions to the nucleic acids were negative although positive reactions were obtained with the nucleic acid-MBSA complex. In parallel, MBSA but not the nucleic acid antigen alone was able to specifically stimulate lymphoid cells *in vitro* of animals immunized with the Poly A-poly U-MBSA complex.

Specific immunological tolerance as manifested by a reduced antibody response could be induced in animals treated with cyclophosphamide 24 hours after priming with the nucleic acid antigen alone and challenged 14 days later with the nucleic acid in adjuvant.

The negative cellular immune response taken together with an antibody response and the production of tolerance suggests that the immune response to this class of compound, and indeed to other simple polymeric substance such as polysaccharides and certain polyaminoacids viz. poly D glutamic acid, may involve bone marrow cells only.

Significance to Biomedical Research and the Program of the Institute:

The findings demonstrate some degree of immunogenicity as well as tolerogenicity of nucleic acids, properties not hitherto clearly ascribed to this class of compound. However NZB and B/W mice (but not other mice) which are characterized by many abnormal immune responses can also respond to this class of materials. Nucleic acids might possibly be categorized therefore, as a class of compounds, perhaps together with other polyanions, as being able to induce antibodies and specific tolerance with no detectable delayed hypersensitivity responses. These data as well as other data from the literature suggest that tolerance at the bone marrow cell level can exist and furthermore that haptenic like materials may be sufficient to induce tolerance at this level. Antibodies to nucleic acids are directly implicated in the pathogenisis of systemic lupus erythematosis and the disease of NZB mice. RNA viruses have been suggested as antigenic stimulus. An understanding of the precise pathways by which animals form antibodies to nucleic acid antigens may help in the therapy of these illnesses in which this class of antibody is involved in the pathology observed. For example, induced specific immunological tolerance to nucleic acids may have a place in the therapy of SLE.

The demonstration of fatal anaphylaxis to nucleic acids in guinea pigs is of immediate importance to those investigators who would use Poly I-Poly-C therapeutically either as an interferon inducer or antitumor agent. It is noteworthy that the supposed non-immunogenicity of Poly I-Poly C is considered by some as a point in favor of its use as an interferon inducer over other compounds having this property.

Proposed Course of Project:

Anamnestic responses to nucleic acids in animals immunized with nucleic acid carrier complexes well as the duration of RNA specific tolerance will be studied.

Publications:

Green, I., Inman, J.K. and Benacerraf, B. Genetic control of the immune response of guinea pigs to limiting doses of bovine serum albumin. Relationship to the PLL gene. PNAS 66: 1267, 1970.

Bluestein, H., Green, I., and Benacerraf, B. The inability of RNA from lymph node cells from immunized strain 2 animals to confer immune competence to strain 13 lymph node cells in the DNP-PLL immune system. Proc. Soc. Biol. & Med. 135:146, 1970.

Ellman, L., Green, I. and Benacerraf, B. Identification of the cell population responding to DNP-GL in lethally irradiated strain 13 chimeric guinea pigs reconstituted with strain 13 bone marrow and $(2x13)F_1$ lymph node and spleen cells. Cellular Immunology I:445, 1970.

Green, I., Ellman, L., Martin, W.J. and Benacerraf, B. Genetic control of specific immune responses in guinea pigs. Cellular Interactions in the immune response. Second Int'l Convocation on Immunology. In press.

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Martin, W.J., Ellman, L., Green, I. and Benacerraf, B. Histocompatibility type and immune responsiveness in random bred Hartley strain guinea pigs. J. Exp. Med. 132:1259, 1970.

Cuatrecasas, P., Green, I. and Illiano, G. Production of anti-glucagon antibodies in Poly-L-lysine "Responder" guinea pigs. Nature New Biology 230:60, 1971.

Selawry, H., Green, I. and Benacerraf, B. Specificity of tolerance to antigens the immune response of which is controlled by the PLL gene. J. of Immunol. 106:276, 1971.

Nussenzweig, V. and Green, I. Lack of correlation between the net charge of antigen and antibodies in guinea pigs immunized with some charged antigens. J. of Immunol. In press.

Benacerraf, B., Green, I., Bluestein, H.G. and Ellman, L. Specific immune response genes. Transplantation. In press.

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Serial No. NIAID - 143
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effector Mechanisms in the Immune Response

Previous Serial No.: None

Principal Investigator: Dr. Ira Green

Other Investigators:
Project Subtitle I: Dr. John van Boxel
Dr. Michael Frank, LCI
Project Subtitle II: Dr. Ethan Shevach, LCI
Dr. Michael Frank, LCI
Project Subtitle III: Dr. Harry G. Bluestein, HMS
Project Subtitle IV: Dr. Ethan Shevach, LCI
Dr. Michael Frank, LCI
Project Subtitle V: Dr. Leonard L. Ellman

Cooperating Units: Harvard Medical School, Department of Pathology
25 Shattuck Street, Boston, Massachusetts

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-9/12	5/12		4-2/12
Professional	1-9/12	5/12		2-2/12
Other	2			2

Project Subtitle I: Lymphocyte Mediated Cytotoxicity

Project Description:

Objectives:

The purpose of this study is to elucidate the mechanisms of cytotoxicity in an *in vitro* system in which either sensitized lymphocytes mediate cytotoxicity, or normal lymphocytes cooperate with heat-inactivated antiserum to mediate cytotoxicity. These *in vitro* models of lymphocyte mediated cytotoxicity may have considerable relevance to mechanisms of *in vivo* delayed hypersensitivity.

Apart from characterizing the phenomenon in terms of specificity, kinetics, requirement for cell contact, etc., efforts have been directed towards elucidating the role of complement if any, and towards identifying the precise lymphocyte class mediating these reactions.

Methods Employed:

Target erythrocytes (RBC) are incubated in tissue culture for 1 - 2 days with guinea pig lymph node cells in the absence of complement. Red cells are radiolabelled with ^{51}Na-chromate and lysis measured by release of ^{51}Cr into the supernatant. Two systems have been studied: 1) sensitized lymphocytes obtained from animals immunized with red cells in complete Freund's adjuvant so as to manifest specific delayed cutaneous hypersensitivity to the target antigen; 2) non-sensitized lymphocytes, similarly incubated with target erythrocytes in the presence of heat-inactivated guinea pig anti-erythrocyte antiserum. Non-sensitized mouse spleen cells and purified human peripheral blood lymphocytes were also investigated for their ability to cooperate with anti-erythrocyte antibody in causing the destruction of target red cells.

Major Findings:

1) Specific target cell lysis occurred in both systems with viable lymphocytes. This required 24 - 48 hours and suggested that the model using sensitized lymphocytes might represent an in vitro manifestation of delayed hypersensitivity. The kinetics of the two systems were different; that associated with heated antiserum and normal lymphocytes being of rapid onset. Both were inhibited by EDTA and irradiation although to a greater degree in the case of sensitized lymphocytes. Studies in which ^{51}Cr labelled RBC of a non cross reacting specificity were placed in the same tube with non labelled RBC sensitized with specific antibody and non-immune lymphocytes demonstrated that this type of lymphocyte mediated cytotoxicity had considerable specificity.

2) Role of complement: a) lymph node cells from guinea pigs genetically deficient in the 4th complement component, in antiserum from C_4 deficient animals and using C_4 deficient guinea pig serum instead of fetal bovine serum, lysed target cells as efficiently as those from normal animals. This system, involving unsensitized lymphocytes cooperating with heated antiserum, was therefore totally deficient in C_4, suggesting that if complement is mediating the reaction it is doing so by way of the late components. b) To determine which class of antibody was required to cooperate with unsensitized lymph node cells in target cell destruction, the guinea pig anti-target cell antiserum was fractionated on DEAE cellulose into the γ_2 and γ_1 classes. These fractions were then used with normal lymphocytes. The results showed that the γ_2 fraction, or complement binding class, and not the γ_1, or non-complement fixing class, mediated the lymphocyte mediated cytotoxic reactions.

3) Lymphocyte class mediating the cytotoxic reaction: a) Macrophages did not seem to mediate the reaction: removal of these cells by glass-bead column filtration did not prevent the remaining cells from lysing target cells in cooperation with heated antibody; unpurified peritoneal exudate cells consisting largely of macrophages were less effective than lymph node cells. b) Thymus derived cells did not seem to be the effector cells in the system in that mouse spleen cells pretreated with anti-θ serum and complement to remove all cells bearing this thymus dependent marker, cooperated with heated antiserum as well as untreated cells.

Significance to Biomedical Research and the Program of the Institute:

Our understanding of the mechanism of cytotoxicity in this system, especially the role of complement and the precise cell type involved, may throw a light on in vivo lymphoid cell mediated cytotoxicity, especially graft and tumor rejection, and may lead to the means for its eventual therapeutic manipulation.

Proposed Course of Project:

1) Role of Complement: a) Red cell membrane fragments will be studied by electron microscopy for characteristic complement mediated "holes", as well as similar examination of cells lysed by sensitized erythrocytes for a possible lesion unique for delayed hypersensitivity. b) Mice and rabbits, deficient respectively in the 5th and 6th components of complement, respectively, will be studied in the same way as the guinea pigs deficient in the 4th component.

2) Cell Type Involved in Cytotoxicity: To ascertain if bone-marrow derived lymphoid cells mediate the reaction requiring heated antiserum, mouse spleen cells will be pretreated with anti-light chain serum and complement to eliminate this population. Other techniques such as cotton column filtration, density gradients and rosette formation techniques will be used to separate and identify cell types involved in the system requiring sensitized lymphocytes.

Project Subtitle II: The Receptor for C'3 on the Surface of Lymphocytes.

Project Description:

Objectives:

A population of lymphocytes has been defined in the mouse which have a cell surface receptor for complement-antigen-antibody complexes. This population corresponds to the population of cells with immunoglobulin determinants on the cell surface, which, in turn, are the bone marrow derived lymphocytes (B lymphocytes). The component of complement responsible for this interaction is presumably C's, or a C's split product. We have repeated these studies to determine if this receptor is present on normal guinea pig lymphoid cells. We have also studied many cell lines derived from malignant human cells, including blast cells obtained from patients with acute leukemia, and malignant lymphoid cells from the mouse.

Methods Employed:

The lymphoid cell population under study is incubated with red cell (E), antierythrocyte antibody (A), complement (C) complexes. Cells which bear the receptor will bind these EAC complexes to their surface and form a rosette. The percentage of rosettes is determined by counting in a hemacytometer.

Major Findings:

Cell populations prepared from guinea pig lymph nodes contain 30% rosette forming cells. No rosettes are seen in preparations of guinea pig thymus. These findings are similar to what has been reported for the mouse. A suspension of L_2C (a transplantable strain 2 guinea pig leukemia) cells contains 80% rosette forming cells. This confirms our other studies which show the L_2C cell to be a member of the B lymphoid cell population. The majority of the cell lines derived from human lymphoid malignancies show 100% rosette formation. This indicates that they too may be homogeneous populations of B cells. About 25% of human peripheral blood lymphocytes form rosettes. The studies on blast cells in human acute leukemia are still in a preliminary stage.

Significance to Biomedical Research and the Program of the Institute:

The receptor for complement-antigen-antibody complexes is an easily identifiable marker of the bone marrow derived lymphocyte. Our studies should indicate if this cell population is involved in certain types of human leukemias and other lymphoid malignancies. If a functionally distinct population of cells is involved in a disease state a specific mode of therapy might be devised which would take advantage of the cells unique physiological properties.

Proposed Course of Project:

The studies on the presence of the complement receptor on lymphoid cells in disease states will be expanded to include immunological deficiency states. The L_2C cell will be used to determine the chemical properties of the surface receptor.

Project Subtitle III: Immunosuppressive Properties of Croton Oil

Project Description:

Objectives:

Croton oil is a promotor or cocarcinogen; the precise mechanism by which corton oil exerts its effect is unknown. Since chemical carcinogens induce tumors that are known to possess "tumor specific antigens" any factor which reduced the immune competence of the host might promote tumor growth. Therefore, croton oil was investigated to see if it had immunosuppressive properties.

Methods Employed:

Guinea pigs were immunized with bovine gamma globulin (BGG) in complete Freund's adjuvant (CFA) containing croton oil. They were also immunized with BGG in CFA in which the active principal of croton oil, phorbol ester, was incorporated in the CFA. The immunosuppressive effect of phorbol ester was also tested when administered at a site distant from the antigen injection.

Major Findings:

Croton oil significantly depressed antibody formation, delayed hypersensitivity and antigen mediated in vitro lymphoid cell stimulation. It was also demonstrated that the immunosuppressive properties of phorbol ester could be reversed by poly I: poly C.

Significance to Biomedical Research and the Program of the Institute:

Croton oil, a cocarcinogen is a powerful immunosuppressive agent and thus part of croton oil's cocarcinogenic property may be ascribed to this property.

These studies again emphasize the importance of intact immunological function (especially delayed hypersensitivity) as a surveillance mechanism for the control of neoplasms.

Project Title IV: General Biologic Properties of the L_2C Cell.

Project Description:

Objectives:

Recent studies have shown that there are two functionally different cell types which participate in the immune response. One cell type is thymus derived or processed; the other differentiates from a bone marrow precursor. The L_2C leukemia cell of the inbred strain 2 guinea pig offers the usual opportunity to study a homogeneous population of lymphocytes and to determine if this cell belongs to one of these functionally distinct subpopulations. These cells can be used as the immunizing source of cells when one is trying to prepare anti lymphocyte subclass specific antisera.

Methods Employed:

The L_2C cell surface antigens have been examined in the living state by means of the fluorescent antibody technique. Multiple anti-guinea pig immunoglobulin and anti-histocompatibility antibodies have been used. The L_2C cell has been cultured in short term tissue culture with ^{14}C lysine to determine if an immunoglobulin is secreted by the cell. Radioimmunoelectrophoresis is then performed on the concentrated supernatants of these cultures. Rabbits have been immunized with both the L_2C cell and with normal guinea pig thymocytes in an attempt to make antisera specific for each cell type.

Major Findings:

Studies with immunofluorescence have shown staining with anti-γ_2, anti-Fab, and anti-L chain antisera. No staining is seen with anti-γ_1, anti-IgM, or anti-IgA. The L_2C cell has a specific immunoglobulin on its cell surface and therefore belongs to the bone marrow derived cell population. The cell surface is also rich in histocompatibility antigen. Studies on the precise class immunoglobulin secretion of these cells and the production of lymphocyte cell type specific antisera are still in the preliminary stage.

Significance to Biomedical Research and the Program of the Institute:

Cooperation between lymphocytes of physiologically different types is essential to the production of most antibodies. The L_2C cell, even though it is derived from a leukemic animal, appears to be an excellent model of the bone marrow derived cell. Further studies of the physiological properties of the L_2C cell should give us a better understanding of the role of the normal lymphocyte in the immune response.

Proposed Course of the Project:

The studies outlined above will be completed. One major goal will be the use of the L_2C cell in the development of antisera against functionally different populations of lymphocytes. These antisera will then be used in studies to define which cell types are cooperating in the immune response.

Project Subtitle V: L_2C Leukemia: Immunoprotection and Immunotherapy

Project Description:

Objectives:

Although leukemia in the mouse has been extensively studied, guinea pig leukemia has received little attention. The only experimental guinea pig leukemia available is a strain 2 specific lymphatic leukemia which arose spontaneously in an inbred strain 2 guinea pig. This leukemia, designated L_2C, has been serially transplanted in strain 2 guinea pigs for over fifteen years. The purpose of the present study was the investigation of the quality and character of the immune response against L_2C which can be induced in strain 2 guinea pigs. A long term goal is the use of this cavian tumor system as a model for the immunotherapy of human leukemia.

Methods Employed:

Immunization protection tests were carried out by immunizing strain 2 animals with large numbers of irradiated L_2C cells intradermally or emulsified in complete Freund's adjuvant and injected in the foot pads. The presence of cellular immunity to the leukemia cells was studied by the delayed hypersensitivity skin reaction, adoptive transfer, and a one-way mixed leucocyte reaction. Humoral immunity was investigated by ^{51}Cr release cytotoxicity assay, indirect immunofluorescence, adoptive transfer and reticuloendothelial clearance of ^{51}Cr labelled L_2C cells. Chemotherapy with cyclophosphamide was attempted. Various approaches to immunotherapy of the leukemia were developed.

Major Findings:

The presence of a strong tumor specific transplantation antigen (TSTA) on the L_2C cells was unequivocally demonstrated by immunization protection tests. A high degree of protection against lethal leukemic challenge was achieved by immunization with irradiated L_2C cells either in adjuvant or following intradermal injection. Unlike the murine leukemia systems, no evidence of humoral antibody to L_2C could be demonstrated. Cellular immunity to L_2C was readily demonstrated and appeared to be the predominant, if not exclusive, factor in the protection observed.

Immunization with irradiated L_2C cells in complete Freund's adjuvant was superior to intradermal immunization with the same number of L_2C cells. Cyclophosphamide has also been found to be highly effective antineoplastic agent. Non-toxic doses are capable of inducing remissions lasting 6 - 8 weeks. Immunotherapy by either active immunization or adoptive transfer of lymph node and spleen cells was shown to be successful when initiated early in the course of the leukemia.

Significance to Biomedical Research and the Program of the Institute:

Leukemia L_2C appears to be an excellent model for the study of chemotherapy and immunotherapy. Our studies in guinea pigs suggest that vigorous immunization of patients with leukemia with autologous tumor cells during periods of remission, when tumor mass is minimal and cellular immunity least impaired, offers a significant chance of successful immunotherapy. Our findings also demonstrate that certain immunization techniques may be more likely to promote development of cellular immunity with little if any humoral, and potentially enhancing, antibody. In the guinea pig L_2C model, immunization with tumor cells in complete adjuvant appears to be superior to intradermal immunization. Various approaches to immunotherapy during periods of cyclophosphamide induced remission are being investigated.

Proposed Course of the Project:

In collaboration with Dr. Katz, Paul and Benacerraf, L_2C immunotherapy and immunoprotection by a novel method of enhancing the immune response to antigens by injecting allogeneic cells is being explored.

Publications:

Bluestein, H., and Green, I. Croton oil induced suppression of the immune response of guinea pigs. Nature 228: 871, 1970.

Ellman, L., and Green, I. L_2C guinea pig leukemia: Immunoprotection and immunotherapy. Cancer (in press).

Serial No. NIAID - 144
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Cellular Basis for Antibody Formation

Previous Serial No.: 144

Principal Investigator: Dr. Bruce Merchant

Other Investigators:

Project Subtitle I:	Dr. John Inman Dr. Gerald Vurek, LTD, NHI Dr. Behzad Mohit, Dept. Clin. Path., CC Dr. Edgar E. Hanna, LMB, NICHHD
Project Subtitle II:	Dr. John Inman Dr. Renee Rouques Dr. Rose Mage
Project Subtitle III:	Dr. Latham Claflin

Cooperating Units: Dr. Joseph Ingraham, Dept. of Microbiology, Univeristy of Indiana Medical Center, Indianapolis, Indiana.
Dr. P. Liacopoulos, Institut D'Immuno-Biologie Hopital Broussais, Paris XIV^{e}, France.
Dr. Zacharie Brahmi, Pasteur Institute, Paris, France
Dr. Thomás Hraba, Czechoslovak Academy of Sciences, Institute of Experimental Biology and Genetics, Na Cvicisti 2, Prague 2, Czechoslovakia.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-4/12			3-4/12
Professional	2-4/12			2-4/12
Other	1			1

Project Description:

Project Subtitle I: Cellular participation in the immune response to chemically defined antigens.

Objectives:

To ascertain whether antibody formation is simply a predetermined differentiative process under direct genetic control or whether it is subject to the influence of random events.

Methods Employed:

Lymphoid cell suspensions from intact and splenectomized azohapten immunized rabbits are assayed for their contained numbers of hapten-specific plaque forming cells (HPFC) directed both against the immunizing hapten and against from eight to twelve structural analogs. The determined activities constitute a profile of antibody-forming cell commitment both with respect to specificity (S) and with respect to immunoglobulin class (C). Thus, these activities graphed alongside one another are termed an SC profile. Sometimes allotype (A) determinations are also performed in which case the profile thus generated is called an SCA profile. Both the SC profile and the SCA profile are characteristic of any given immune cell suspension and may be used to identify it.

In splenectomized rabbits immunized intravenously the SC profile is determined in each of four marrow cell suspensions, those derived from the right and left femurs and from the right and left tibias. This permits bilateral comparison of the status both of the femoral and of the tibial SC profiles present in the rabbit at the moment of sacrifice. If antibody formation is a simple differentiative process under strict genetic control, the SC profiles of two large anatomically equivalent lymphoid organs (e.g. two femoral marrow spaces) should be quite similar, if not identical. This would be the prediction of the germ-line theory of antibody formation interpreted in its strictest and most rigorous sense.

Major Findings:

1. The bone marrow is an important (perhaps the major) site of active γG antibody formation in the rabbit. In this important respect, the rabbit may more closely resemble humans than do other common laboratory animals such as guinea pigs or mice.

2. The SC and SCA profiles in any given rabbit may differ markedly from a right-hand to a left-hand marrow regardless of whether the right to left comparison is for femurs or for tibias. This indicates that antibody formation is subject to the influence of random events. These finding argue against a strict interpretation of germ-line theory.

3. Marked differences in bilateral SC and SCA profiles are most notable early (6-8 days) following primary immunization. Bilateral SC and SCA profiles obtained following secondary immunization (10-14 days) show less notable differences, and bilateral profiles examined after tertiary immunization (20-60 days) commonly resemble one another closely. These findings are consistent with the view that increased and cumulative numbers of random events occur with increased time. If the SC profile generating events in

each of two marrow spaces were completely independent, one of the other, and if these events were strictly random, given the common and equal antigenic stimulus provided and a sufficiently large number of random events (i.e. sufficient time) the appearance of the two independent profiles would ultimately be expected to converge. It is gratifying that this prediction is consistent with our observations; however our observations in no way diminish the importance of genetic information pre-existing in the precursors of immunocompetent cells.

4. Present findings do not as yet indicate the nature of the random events contributing to the generation of antibody specificity. These events could be at the levels of differentiative determination, maturation, proliferation or even cell migration. Alternatively, random events could be operating at the critical level of genetic direction; the latter would imply somatic rearrangement or mutation as a process in the generation of antibody diversity.

Significance to Biomedical Research and to the Program of the Institute:

Strict germ-line theories of immunity essentially imply that all normal immunologic events are genetically directed. If true, it follows that autoimmune diseases are either congenital or are the result of an intervening abnormal or pathologic process which permutes normal immune responses. In contrast, selective theories of immunity depend on the "tyranny of chance" (i.e. random events) to provide for much or most of the specificities comprising a normal immune potential. Our present findings emphasize the normal importance of chance events. Naturally, if one seeks to control autoimmune diseases, it is essential to understand as much as possible about the processes, normal or abnormal, responsible for their initiation.

Proposed Course of Project:

Present studies will be amplified by extending them to irradiated hosts reconstituted with a precharacterized (SCA profile determined) infusion of autologous marrow cells. Efforts will be made to perform similar experiments in inbred species (guinea pigs and mice) in order to ascertain whether our present findings represent a general biologic phenomenon or a special one particular to rabbits.

Project Subtitle II: Specific and Quasi-Specific Antibodies and Their Relation to the Traditional Concept of the Immune Response

Objectives:

1. To identify the character and specificities of the so-called "nonspecific" new immunoglobulins which are produced concomitant with antibodies and which commonly predominate over immunoglobulin-specific for the immunogen early following immunization.

2. To ascertain whether modifying the structural nature of an immunizing hapten will result in a change in the character both of specific and of "non-specific" immunoglobulin production.

Methods Employed:

The basic experimental and assay methods used are essentially the same as those outlined under Subtitle I above. A major feature of this work is that it employs tripeptide "spacers" subjacent both to the immunizing azohapten and to the assay azohapten and its various analogs on the dozen different indicator erythrocyte systems comprising our SC assay profile. These dozen unique azohapten-tripeptide molecules of known structure, uniformity and sequence were planned and synthesized by Dr. John Inman. Our main immunizing haptens have been p-azobenzenearsonate, p-azobenzenesulfonate, and p-azophenyl each separately and covalently linked to acetyl-tyrosyl glycyl glycine which is then coupled via an azide group to the epsilon amino residue of lysines on either duck erythrocyte membranes (our immunogen) or the surface of sheep erythrocytes (our indicator system). We have also done some experiments with dimethylaminonaphthalenesulfonate coupled similarly via a β-alanyl glycyl glycine tripeptide.

Definitions - Our findings have necessitated the introduction of new terms and explicit definition of other terms. We define antibodies as all intact functional new immunoglobulin molecules elicited by an antigenic stimulus. Antibodies may be either specific or quasi-specific for the immunizing antigen (or hapten). Antibodies reacting more strongly with the immunizing hapten than with any other test hapten are termed specific antibodies and they are identical with traditional antibodies. Other new immunoglobulin molecules more strongly reactive with a structural analog than with the immunizing hapten are termed quasi-specific antibodies or "Quasibodies" for short.

Major Findings:

In the immune response of rabbits to azohapten and azohapten-tripeptide antigens:

1. Large portions of the "non-specific" immunoglobulins accompanying a specific antibody response are actually quasibodies more avid for an analog of the immunizing hapten than for the hapten itself. Many cells release quasibodies which show no detectable reaction with the haptenic structure which elicited them!

2. Quasibodies may be either of the IgM or of the IgG class.

3. Total cells releasing IgM quasibodies are normally far more numerous than total cells releasing specific IgM antibodies.

4. Cells releasing IgM quasibodies best reactive with only a single analog test hapten occasionally outnumber the cells releasing specific IgM antibodies at the same point in time.

5. This finding is especially prominent in early immune responses.

6. Cells releasing IgG quasibodies are prominent in the early stages of the IgG response.

7. Cells releasing IgG quasibodies are relatively scarce within a week or two after the onset of new IgG immunoglobulin secretion. By this time they have largely been replaced by cells releasing conventional specific antibodies. Accordingly, quasibodies coexist with specific antibodies but since IgG immunoglobulin production normally soon predominates in immune responses (7-10 days), conventional specific antibodies soon become dominant thus accounting for the effective specificity normally displayed in antibody formation.

8. In normal animals, the onset of IgG antibody formation can be delayed from one to three weeks simply by presenting an azohapten "spaced-out" from its macromolecular carrier by a tripeptide of known sequence. Presumably the carrier molecule's contribution to precursor cell activation has been minimized by this device.

9. In the interim, IgM quasibody and specific antibody formation continue to progressively increase, commonly reaching very high levels. For example, cells releasing specific IgM antibodies commonly number about $2000/10^6$ nucleated cells while roughly equal numbers of cells releasing IgM quasibodies may be found for six or more of the twelve standard structural analog test systems. Thus, the IgM immune commitment at that point is about 2000 cells releasing specific antibodies and at least 12,000 cells releasing quasibodies of various specificities per million nucleated spleen cells.

10. These findings strongly reinforce our earlier findings with the replica-immune plaque assay which we interpreted as indicating that the IgM and the IgG secreting immune cell systems are effectively independent and autonomous.

11. When IgG production finally supervenes it may at first be either predominantly specific or predominantly quasispecific. In any event, it ultimately becomes specific within a few days or weeks. These findings provide a rationale for the evolutionary ascension of IgG over IgM production.

12. Our experiments have disclosed the heretofore unappreciated existence of quasibodies partly because of their comprehensive nature, but mainly because our assay systems range generally between a thousand and a million fold more sensitive than most standard immunoserologic assay systems in current use.

Significance to Biomedical Research and to the Program of the Institute:

If we are to deal with and ultimately control auto-immune phenomena it is essential to understand the events required for initiation of "improper" or "inappropriate" immune responses. Because normal immune responses are ultimately and effectively specific, immunologists have generally assumed that immune precursor cell activation is normally a highly specific process. Our present findings show that such a simplistic assumption is grossly incorrect. Normal antibody responses are at first predominantly "inappropriate" becoming mainly and traditionally specific only after the onset of IgG synthesis and with the passage of sufficient time. Because initially "inappropriate" immune responses are the normal rule rather than the exception, this automatically imposes an entirely new frame of reference on all basic concepts about the pathogenesis of humoral autoimmune diseases. Thus, it is time for a complete rethinking both of our strategy and of our tactics aimed at the prevention and control of autoimmune diseases.

Proposed Course of Project:

Present lines of experimentation will be continued in the rabbit. Strong efforts are already being made to extend these types of studies to guinea pigs and mice. If successful, experiments will be conducted in a strain of mice (NZB) known for its propensity to develop multiple autoimmune diseases in order to determine, if possible, the nature of the "lesion" or flaw in its immune mechanism.

Project Subtitle III: Lymphoid Cell Transformation with Immunogenic or Plasma Cell RNA

Project Description:

Objectives:

Recent investigations have demonstrated the involvement of RNA in the transfer of immunological information; RNA involvement has been implicated in antibody synthesis, homograft rejection and delayed hypersensitivity. The idea of specific messenger RNA is disputed by the appearance of traces of antigen in RNA preparations. In an attempt to circumvent this problem, attempts were made to determine whether RNA extracted from a myeloma tumor (i.e. not produced in response to antigen injection, therefore containing no antigenic moieties) could transform normal lymphoid cells. If successful, a study of the RNA and its characteristics would be undertaken.

Methods Employed:

A homogeneous myeloma tumor, MOPC 315, which produces an IgA immunoglobulin with high binding affinity for dinitro- and trinitrophenyl ligands was chosen for this study. RNA was extracted from the tumor cells after the methods of several investigators, analyzed by density gradient ultracentrifugation, and tested in various *in vitro* culture systems for its ability to confer upon non-immune spleen cells or lymph node cells the capacity to secrete myeloma specific immunoglobulin. A search for such transformation was made at the cellular levels employing a hemolytic plaques-in-gel assay technique that detected TNP-specific plaque forming cells.

In addition, "immunogenic" RNA was extracted at different intervals after immunization of mice with sheep erythrocytes and incubated in vitro with normal spleen cells or lymph node cells. The ability of this RNA to cause normal lymphoid cells to secrete anti-erythrocyte antibody was also determined using the hemolysis-in-gel assay.

Major Findings:

Although cell cultures were exposed to a wide range of concentrations of tumor or of immunogenic RNA, utilizing various culture media and incubation conditions, no evidence for transfer of immunologic information was obtained. Experimental controls demonstrated no significant loss in viability or in background activity to the TNP ligand.

Significance to Biomedical Research and to the Program of the Institute.

Since the myeloma tumor cells employed afforded the most uniform and homogeneous source of messenger-RNA that one would expect to encounter with a mammalian cell system, and since the sucrose gradient ultracentrifugal profiles indicated that most messenger RNA remained undegraded, it seems likely that the positive cases of RNA transformation reported in the literature may have depended on some factor other than messenger RNA.

Proposed Course of Project:

No further studies into the role of RNA in the immune response are currently planned.

Publications:

Merchant, B. "Comet Plaques" evidence for storage of low efficiency hemolysins by indirect plaque-forming cells. J. Immunol., 104: 1565-1568, 1970.

Hanna, E.E. and Merchant B. Secretion of monospecific anti-hapten IgG antibodies by rabbit immunocytes. J. Immunol. In press.

Serial No. NIAID - 145
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Relationship Between Antigen Structure and Characteristics of the Immune Response

Previous Serial No: 149

Principal Investigator: Dr. John K. Inman

Other Investigators:		
	Project Subtitle I:	Dr. Bruce Merchant
	Project Subtitle II:	Dr. William J. Martin Dr. John R. Wunderlich, NCI Dr. Rose G. Mage
	Project Subtitle III:	Dr. William E. Paul Dr. Joseph M. Davie Dr. Jack Stobo Dr. Behzad Mohit, CC, NIH

Cooperating Units: Dr. John R. Wunderlich, Immunology Branch, NCI
Dr. Behzad Mohit, Dept. of Pathology, NIH Clinical Center

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2-10/12			2-10/12
Professional	1-10/12			1-10/12
Other	1			1

Project Description:

Objectives:

1) To study the development of immunological specificity during various stages of humoral immune responses elicited by immunogens having very large, uniform, structurally defined determinants (or haptenic groups).

2) To discover and define essential structural features of immunogens pertaining to either carrier or haptenic functions and to determine how these features are interrelated.

3) To discover what structural characteristics of antigens cause their immunogenicity to be controlled by certain genetic factors.

4) To learn how specific immune responses (cellular and humoral) can be modified or regulated by chemical manipulation of the immunizing antigen.

Project Subtitle I: Cellular Studies on the Development of Humoral Immune Responses Directed Toward Large, Synthetic Haptens

Methods Employed:

The haptens were synthesized as C-terminal tert.-butyloxycarbonyl hydrazides of the following structures: various substituted derivatives of phenylazo-N-acetyl-L-tyrosylglycyglycine, and N-terminal dinitrophenyl, trinitrophenyl and dimethylaminoaphthalenesulfonyl derivatives of β-alanylglycylglycine. The purified compounds were stable and were converted by convenient procedures to reactive acyl azides just prior to coupling with test red cells, or with the duck red cell stromata used for i.v. immunization. Optimal conditions (time, pH, concentration, buffer composition) for coupling were found.

Conventional methods of peptide synthesis were employed. Compounds were isolated by differential extraction, and ion-exchange and adsorption chromatography. The purified hapten precursors were characterized by chromatographic behavior, functional group tests, absorption spectra, and, in some cases, elemental and amino acid analyses.

Major Findings:

Studies were carried out in collaboration with Dr. Bruce Merchant. Procedures were developed for covalently attaching large haptenic groups to the surfaces of intact red cells. The resulting cells were used in hemolytic plaque assays to detect hapten-directed antibody producing cells using techniques developed by Dr. Merchant. The main purpose of the work was to study the development of clonal heterogeneity during immune responses to haptens of uniform and defined structure. Haptens were desgined and synthesized so that they would be expected to fill the combining sites of antibodies or of receptors on antibody producing cells or their precursors. Contributions of carrier structures to the haptenic function can in this way, at least, be minimized. Thus, a clearer picture of the intrinsic heterogeneity of the responses should be obtained than could be afforded by use of the small haptens commonly employed in immunological research. To this end, a group of about fourteen derivatized tripepties were synthesized in this laboratory and coupled as necessary to red cells used for assays. Three of these haptens were conjugated to a suitable immunizing carrier.

Major findings are summarized in part below. In the early stages of an immune response in rabbits, it was common to observe many direct plaque forming cells (PFC) from spleen tissue by employing test haptens other than the immunizing one. The best haptens included some having similar and some having dissimilar structures to the immunizing hapten. Numbers of such plaques were often of the same order of magnitude as those observed with the homologous hapten, and, in a few instances, they were significantly more numerous. Assays carried out with mixtures of hapten-conjugated sheep red cells (or of sheep and nucleated pigeon test cells), and studies with multiple conjugated test cells indicated that most of these populations of PFC producing anti-hapten antibodies did not share members and therefore did not produce cross-reacting antibodies in the usual sense; that is, they produced antibodies that combined somewhat better with an alternate hapten than with the immunizing one. This phenomenon was found to extend to indirect (γG type) plaque forming cells in the early primary response. Many weeks following immunization, a typically high degree of specificity was found with the predominantly indirect PFC. Cross-reactions that did occur then were mostly or exclusively of conventional type (i.e., PFC reacted with both immunizing and alternate haptens).

In some rabbits conversion from primarily γM to mainly γG PFC in the primary response occurred considerably later when animals were immunized against the large haptens as compared with smaller ones. Considerable numbers of PFC were found in rabbit bone marrow tissue as well as in spleen during the early immune response.

Project Subtitle II: Chemically Modified and Synthetic Antigens: Special Effects on Immune Responses

Methods Employed:

The large haptens were coupled to proteins (BSA and BGG) via C-terminal acyl azide intermediates. Results are being evaluated by free zone electrophoresis and isoelectric focusing in polyacrylamide gels with the aid of radioiodinated free hapten derivatives.

Major Findings:

A joint project with Dr. Martin in our laboratory and with Drs. Wunderlich and Freeman Fletcher of NCI was completed and published recently under the title "Enhanced Immunogenicity of Chemically-Coated Syngeneic Tumor Cells" (see publications). Samples of several transplantable tumor lines in inbred mice were irradiated, treated chemically with one or two reagents, and injected into syngeneic mice. Lymphocytes taken later from these mice exhibited significantly increased cytotoxic capacity toward the corresponding tumor cells in culture (Dr. Wunderlich). The two modifying reagents were the carbohydrate-binding protein, concanavalin A (binding non-covalently), and N-hydroxysuccinimide ester of 6- (2,4-dinitrophenylamino) caproic acid (binding covalently). Investigation of other modifying reagents is in progress.

The antibody responses in rabbits to protein and polymer conjugates of substituted azophenyltripeptidyl haptens (See Subtitle I, Methods) are being followed by Dr. Mage and myself. A search is being made of individual rabbits which produce antibodies of markedly restricted heterogeneity.

The aim is to see if large, "site-filling" haptens increase the frequency of responses of this type. Antibody isolated from restricted responders could provide material useful for amino acid sequence studies.

Project Subtitle III: Interactions of Cell Surface Receptors with Proteins and Haptens Bound to Polymer Matrices.

Methods Employed:

The fragment condensation approach using N-acylpeptide azides is being used to prepare intermediates for cell receptor topology studies.

Haptenic groups were bound covalently through either bonds to hydroxyl-containing polymers (used by Dr. Mohit) employing epoxides under either basic or Lewis acid catalysis. Haloalkyl groups introduced in this way are converted to amino functions and conjugated with a reactive hapten intermediate.

Major Findings:

Derivatives of large, highly cross-linked polyacrylamide beads (see previous annual report) have continued to show promising results in the fractionation of cell suspensions with respect to specific surface receptor properties (Drs. Stobo and Paul). Work is being planned(with Drs. Davie and Paul) for exploring certain topological features of cell receptors using specially designed bi- and poly- functional reagents. Synthesis of intermediates leading to these reagents has been started.

Methods of partitioning immunocompetent cells in aqueous two-phase systems are being investigated in collaboration with Dr. Behzad Mohit. High molecular weight polymers with covalently bound haptenic groups were prepared in this laboratory. Dr. Mohit has found that these substances apparently bind specifically to cell surfaces and noticeably change the distribution of bound cells between liquid phases and interfaces. Further work is in progress.

Significance to Biomedical Research and the Program of the Institute:

Progress made toward the stated objectives will be applied to increasing the general knowledge and understanding of many aspects of immune responses at the molecular and genetic level. Knowledge of this sort may lead to a rational approach to modify or control immune responses which act as a curative or etiologic factor in disease involving autoimmunity, infection, homograft rejection, allergy and malignant cell growth.

Proposed Course of Project:

Work will continue on a collaborative basis in the three subtitled areas. Many interesting leads have turned up in areas such as the developmental sequence of immunological specificity in humoral immune responses and in the modification (enhancement) of cellular immunogenicity toward tumor-specific antigens. Such leads will be followed with particular interest.

Serial No. NIAID - 146
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical, Genetic and Immunochemical Studies of Immunoglobulins: Use of Genetic Markers on Immunoglobulins to Study Gene Expression, Genetics of Antibodies and the Immune Response

Previous Serial Number: 148

Principal Investigators: Rose G. Mage, Ph.D. and Glendowlyn O. Young-Cooper, M.S.

Other Investigators:

Dr. A. Chersi	LI-NIAID
Dr. J. Winfield	LI-NIAID
Dr. M. Goldman	LI-NIAID
Dr. W. Paul	LI-NIAID
Dr. J. Davie	LI-NIAID
Dr. W.J. Martin	LI-NIAID
Dr. E. Appella	LBGY-NCI
Dr. K. R. McIntire	LBGY-NCI
Dr. J. Pincus	LM-NIDR

In addition, collaborative experiments have recently been started with Dr. J. K. Inman, LI-NIAID. We provide rabbits of defined allotype to Dr. E. B. Merchant, LI-NIAID.

Cooperating Units: Dr. S. Dubiski, The Toronto Western Hospital, Toronto, Canada
Dr. S. Dray, University Illinois Medical Center, Chicago, Illinois
Dr. S. Tosi, Osservatorio Di Genetica Animale, Torino, Italy
Dr. G. Siskind, Cornell Univ. Med. Center, N.Y., N.Y.
Dr. J. Cebra, Johns Hopkins Univ., Baltimore, Md.
Dr. A. Lawton and Dr. M. Cooper, Univ. of Alabama Med. Center, Birmingham, Alabama

In addition, this year, the following investigators have used rabbits of defined allotype which we have supplied and/or have received specific reagents which we have prepared: Dr. C. Bell, Chicago, Ill., Dr. S. Sell, LaJolla, Calif., Dr. B. Wolf, Phila., Pa., Dr. J. Young, Berkeley, Calif., Dr. E. Haber, Boston, Mass., Dr. M. Freedman, Toronto, Canada, Dr. Asher Frensdorff, Tel Aviv, Israel, Dr. Jose O'Daly, Baltimore, Md., Dr. W.J. Mandy, Austin, Texas, Dr. R. Martineau, Rochester, N.Y. and Dr. A. Nisonoff, Chicago, Ill. We have also typed samples for various investigators at the NIH, around the U.S. and around the world.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	5-3/12			5-3/12
Professional	4-3/12			4-3/12
Other	1			1

Project Description:

Objectives:

To define by combined immunological and chemical approaches, the chemical nature of structural differences between genetically controlled polymorphic forms of immunoglobulins and between different classes, subclasses, types and subtypes of their polypeptide chains. To utilize this information about the location and chemical nature of the markers on various chains in performing and analyzing breeding studies. To utilize reagents specific for the various chains and markers in studies which involve transfer of cells from donor to recipient rabbits, in affecting the phenotypic expression of alleles or chain types, and in studies of antigen-binding receptors on cells. All of these approaches have as their overall objective, the definition of the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses.

Methods Employed:

Rabbits of known allotype are bred:

1) To produce homozygous rabbits of all combinations at three or more genetic loci.

2) To produce heterozygotes for use in specific experiments and as F1 progeny in linkage studies.

3) To produce F2 and backcross progeny in linkage studies.

4) To produce allotype-suppressed rabbits.

5) To study the genetics of the immune response to Pneumococcal vaccines, DNP-Protein conjugates, and other antigens.

Sera of rabbits from the categories outlined above are collected. The sera are typed by gel diffusion and hemagglutination inhibition methods. The

Bovine serum albumin (BSA) taken up by thymus cells from a donor differing by 2 or 3 allotypic markers (a-locus, b-locus and A14/A15) from recipient rabbits is immunogenic when administered to newborn rabbits. The same dose of antigen (15 μg) as well as larger doses, administered in solution is usually tolerogenic. Recipients of thymus cells develop detectable levels of donor type immunoglobulin markers persisting for 8-10 weeks. There appears to be no relationship between the presence of donor type immunoglobulin and the antibody response. No evidence for information transfer between donor and recipient cells was obtained. The antibody appeared to be exclusively of host origin. Cellular chimeras in the peripheral blood were not demonstrated using sex chromosomes as markers. (Work of Dr. J. Winfield and W. J. Martin).

Peripheral blood leukocytes from rabbits which were heterozygous for markers on their immunoglobulin light chains (b^5/b^9) were maintained in vitro for up to 24 hours in the presence or absence of antibody to b9. After culture, they were transferred into lethally irradiated b^4/b^4 hosts. Recipients of cells exposed to antibodies to allotype markers showed a striking increase in concentration of circulating b9 molecules and number of b9 plasma cells in their spleens compared to control animals receiving untreated cells from the same donor. (Work of Dr. J. Cebra et al., Johns Hopkins).

In order to carry out all the studies outlined above, we breed and maintain large numbers of rabbits in our own colonies. We produce and characterize large volumes of anti-allotype antisera made with a variety of donor-recipient combinations.

Significance to Biomedical Research and the Program of the Institute:

These studies help to define the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses. They contribute to our understanding of the complexity of mammalian genetic systems, the chemical basis for specificity of protein antigens and antibodies and the nature of structural genes for immunoglobulin polypeptide chains. Numerous direct homologies have been found between rabbit and human immunoglobulins. The detailed genetic analysis which can be done by breeding rabbits can be applied to the human system. Rabbit allotypes are also analogous in many respects to cellular isoantigens involved in transplantation, leukocyte groups and some blood cell groups. An understanding of the factors which control genetic expression of these isoantigenic types is thus of broad fundamental interest.

Proposed Course of the Project:

We are continuing most of the studies described. We will continue to breed and search for additional examples of recombination between V_H and C_H genes. We are breeding to obtain the already discovered recombinant form of chromosome in homozygous state so that structural comparisons of the original and the new form of heavy chain can be conducted. Work is in progress on the amino acid sequences of both variable and constant region cysteine-containing peptides from kappa and lambda-type light chains of different allotypes. Work sera are used as sources of IgG and light and heavy polypeptide chains for chemical sequence studies and for immunizations of rabbits and other animal species.

Rabbits of defined allotypes are immunized with IgG, light chains, or other antigens such as lysozyme, pneumococcal vaccines, hapten-protein conjugates and (for idiotype studies) purified antibodies or pneumococci coated with anti-capsular antibodies. Antisera are tested by qualitative and quantitative immunologic and electrophoretic techniques.

Allotypes, types, subtypes and idiotypes are quantitated by: 1) radial immunodiffusion, 2) hemagglutination inhibition, 3) quantitative precipitation of purified ^{125}I-labeled proteins with specific antisera, 4) binding of labeled proteins to cross-linked antisera (immunoabsorbents), 5) inhibition of binding of labeled antigens to cross-linked antisera. Immunoabsorbents are also prepared and used to purify antibodies and antigens of known specificity.

Newborn rabbits which are offspring of: 1) normal mothers, 2) mothers immunized against a paternal allotype, 3) mothers immunized against an allotype or allotypes carried in the genotype of implanted zygotes, 4) allotype-suppressed mothers, are injected with anti-allotype antisera in order to suppress expression of allotypes or chain types or subtypes. In analogous experiments, newborn mice are injected with purified rabbit anti-mouse κ-type L chain antibodies.

Purified light and heavy chains, or fragments of these are obtained from IgG of normal sera or specifically purified antibodies of known allotype, as well as from sera of allotype-suppressed rabbits. These are studied using a large variety of methods for sequence determination including automated analysis with a Beckman sequencer.

Cells from rabbits of defined allotype are used in transfer studies. Fetal liver (with A. Lawton and M. Cooper), thymus cells (with J. Winfield and W. J. Martin) and peripheral blood cells (with J. Cebra, et al.)have been administered to either irradiated or newborn rabbits. The donor and recipient cells and their products are then studied by a variety of histological and immunological techniques.

Major Findings:

We are studying the linkage of genes for heavy chain variable regions (V_H- a locus) to C_H genes (e.g. A14 and A15). The markers of the a locus are closely linked to the A14 and A15 markers on rabbit C_γ chains. One apparent recombinant has been observed among 216 progeny of double backcross matings. The finding of a recombinant in this relatively small number of progeny is at least suggestive that the distance between the a locus and the gene for the C_γ region in rabbits is greater than the distances between the genes for the C_γ, C_η and C_α regions in mice since no recombinants between markers on these regions have been observed among more than 2800 backcross progeny.

The presence of threonine in the Cγ2 region (position 309;Eu numbering) correlates with the A14 allotype and the presence of alanine at this same position correlates with the A15. A high degree of homology of constant region domains of rabbit and human γ chains have been observed (~ 60-80%).

A comparison of amino-terminal amino acid sequences of normal and antibody L chains of various allotypes showed that, when aligned by homology with human and murine kappa chains, there are three chain lengths with deletions at the amino-terminus ($V_\kappa I$, $V_\kappa II$, $V_\kappa III$). The majority of L chains of b4 and b5 allotype have $V_{\kappa II}$ length. The b6 light chains have predominantly $V_{\kappa I}$ length, and a large portion of b9 chains are unique in having valine at position 1 and $V_{\kappa III}$ length. These data suggest that at least some V_κ genes of rabbit light chains can be associated with any of the 4 alleles but expressed in different proportions in each allotype.

A unique sequence for 21 residues was obtained for a purified anti-benzene-arsonate antibody of restricted heterogeneity from a rabbit of b5 allotype. An unusual residue, Ala at position 8 was observed. A sequence almost identical to that of the antibody L chains (including the unusual Ala) was found in normal kappa chains from another non-immunized rabbit which was recovering from total suppression of b5 (kappa) allotype. This suggests that the light chains produced by a recovering suppressed rabbit may be restricted in heterogeneity and not representative of the normal range of immunological reactivity of the animal. The rabbit which produced the anti-hapten antibody of restricted heterogeneity lived for more than 3 1/2 years and produced anti-benzene-arsonate antibody at various times over a 33 month period. We have prepared anti-idiotype antisera to purified antibodies made at four different stages. Some antibodies produced as much as 28 months apart, appear to share idiotypic specificities. We have also prepared anti-idiotype antisera against several anti-pneumococcal antibodies of restricted heterogeneity. A rabbit which was boosted with the same vaccine one year after it initially made anti-SVIII antibody of restricted heterogeneity produced a somewhat less homogeneous population of antibodies at that time. Anti-idiotype antibody prepared against the initial antibody did react strongly with the antiserum obtained after the boost.

A series of pedigrees have been developed (with Dr. Pincus) which show that the ability to respond to pneumococcal vaccine under the conditions of immunization used, with a response of restricted heterogeneity has a genetic basis. The trait does not appear correlated with allotypes of the b locus since we now have rabbits with all b-locus allotypes carrying the trait and producing antibodies of restricted heterogeneity. Such antibodies will be valuable adjuncts to studies in progress on structure of light chains of different b-locus allotypes.

Similarly, for studies of lambda chains, we have obtained rabbits fully suppressed for expression of kappa chains which also lack c7 a) because of the genotype chosen, and b) because of suppression of the c7 allotype (lambda) along with the b5 allotype (kappa). These sera will be useful for determining the structural differences between lambda chains with c7 and c21 markers.

is in progress attempting to obtain additional anti-hapten and anti-pneumococcal antibodies of restricted heterogeneity. We are studying the sera of relatives in pedigrees of "restricted responders" using anti-idiotype reagents. We plan to use the system of transfer of cells to irradiated recipients in additional studies of the mechanism of allotype-suppression, as well as for other investigations (restricted antibody production, possibility of control genes for immunoglobulin structural gene-loci).

Honors and Awards:

Invited lecturer, conference on Immunoglobulins, New York Acad. Sciences, March, 1971. Invited lecturer, International Congress of Immunology, August 1971. Also workshop co-chairman (Allotypes).

Publications:

Landucci-Tosi, S. and Mage, R. G.: A method for typing rabbit sera for the A14 and A15 allotypes with cross-linked antisera. J. Immunol. 105: 1046-1048, 1970.

Frensdorff, A., Jones, P., Berwald-Netter, Y., Cebra, J. and Mage, R.: Selective stimulation of allelic expression: Effect of anti-allotype on lymphoid cells. Science 171: 391-394, 1971.

Mage, R. G., Young-Cooper, G. O. and Alexander, C.: Genetic control of variable and constant regions of immunoglobulin H chains. Nature 230: 63-64, 1971.

Mage, R. G.: Normal and altered quantitative expression of allotypes on light and heavy chains of rabbit immunoglobulins. Annals N.Y. Acad. Sci. In Press.

Appella, E., Chersi, A., Mage, R. G. and Dubiski, S.: The structural basis of the A14 and A15 allotypic specificities in rabbit immunoglobulin-G. Proc. Nat. Acad. Sci. In Press.

Mage, R. G.: Structural localization, allelic exclusion and linkage relationships of rabbit allotypes. In Amos, D.B. (Ed.): Progress in Immunology. New York, Academic Press. In press 1971.

Serial No. NIAID - 147
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunogenetics of Mouse Immunoglobulins

Previous Serial No.: 147

Principal Investigators: Miss Rose Lieberman

Other Investigators:
Project Subtitle I: Dr. Michael Potter, LBGY, NCI
Project Subtitle II: Dr. Michael Potter, LBGY, NCI
Project Subtitle III: Dr. Charles Janeway, LI, NIAID

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3			3
Professional	2			2
Other	1			1

Project Subtitle I: Study of Immunoglobulin Determinants in Mice with Homologous Antisera to Determine the Genetic Control of the Structure of Immunoglobulin Molecules

Project Description:

Objectives:

1. To prepare homologous anti-allotypic and idiotypic (myeloma-specific) antisera highly specific for determinants on various parts of the different chains of mouse immunoglobulin molecules.

2. To study genetic control of immunoglobulin determinants.

3. To use genetically controlled immunoglobulin markers in studies of antibody synthesis.

4. To determine if identical molecules, based on biologic activity and identification of Fab determinants, can be found in myeloma proteins

from independently induced plasma cell tumors.

Methods Employed:

1. Preparation of immunoglobulin immunogens (Fc, Fab and light chains) from normal and plasma cell tumor bearing inbred mice.

2. Immunization of inbred strains of mice with specific normal or myeloma immunoglobulin molecules or fragments of immunoglobulin molecules (chains).

3. Identification of immunoglobulin determinants with homologous antisera using immunoelectrophoresis, Oudin and Ouchterlony gel diffusion methods, radioisotope tagging, precipitin inhibition method, hemagglutination inhibition techniques and immunoabsorbants.

4. Selective breeding of mice to determine linkage, allelism, independent assortment and crossover of genes. Production of coisogenic stains to propagate and isolate genetic markers in a known genetic environment. Chromosome mapping of genetic markers of immunoglobulin determinants with other genetic markers as coat color, etc., to facilitate ease of identification of immunoglobulin genetic markers and also to determine the location on the chromosomes of genes controlling specific immunoglobulin determinants.

5. Development of techniques employing immunoglobulin determinants as genetic markers in problems of transplantation, histocompatibility, transfusion reactions, immunization and antibody synthesis.

Major Findings:

Immunoglobulin determinants have been identified on the heavy chains of immunoglobulin molecules in the mouse. Six heavy chain γG (γ2a), three heavy chain γH (γ2b) and four heavy chain γA determinants have been identified. These determinants have been found to be closely linked in their genetic control. In addition, three other determinants which have thus far not been assigned to specific immunoglobulin molecules have also been identified. The 16 determinants are present in the Fc region (constant region) of the heavy chain. In addition two γF alleles have been identified by the differences in the electrophoretic mobility of their Fc fragments. Nine heavy chain linkage groups have been identified; six in inbred strains and three additional ones in wild mice.

Immunoglobulin determinants in other regions of immunoglobulin molecules, namely the Fab region, have also been identified for different classes of immunoglobulins. These have been identified with homologous precipitating antisera prepared with specific myeloma proteins. These antisera identify myeloma specific determinants (MSD) or idiotypic determinants that are restricted to the Fab region of the myeloma protein used as the immunogen.

Many of the myeloma proteins, specifically IgA's have been found to have biologic activity, i.e., they have been shown to combine with Pneumococcus C polysaccharide, Salmonella polysaccharide, dextran, 2,4-dinitrophenyl group (DNP), 2,4,6-trinitrophenyl groups (TNP), etc.

Recently, 8 plasma cell tumors, induced independently in genetically similar inbred mice (BALB/c), produced IgA myeloma proteins all of which combined with phosphoryl choline, which is found in the C polysaccharides of Pneumococcus and Lactobacillus. Homologous antisera prepared to these 8 functionally similar myeloma proteins showed that five of these (M299, H8, T15, S63 and S107) have a common antigenic determinant which was found to be on the Fab portions of the respective myeloma proteins. This common antigenic determinant was not found on the light or heavy chains alone but was only present on the Fab or on preparations of recombined light and heavy chains from the respective myeloma proteins indicating that this was a conformational determinant derived from the interaction of the light and heavy chains. Sequence studies of the amino terminus of the light chains of T15 and H8 indicate that the first 21 residues of both proteins are identical.

Homologous antisera to the three remaining functionally similar proteins (M167, M511 and M603) identified myeloma specific determinants that so far are unique to each separate myeloma protein used for immunization. Amino acid sequence studies of the light chain of two of these (M167 and M603) showed that there were differences between these two proteins as T15 and H8, and between each and beginning with the 9th residue.

Significance to Biomedical Research and the Program of the Institute:

It is difficult to study the genetic control of the diversity of antibody using normal antibodies since these are mixtures of many types of molecules. Myeloma proteins are pure molecular forms of immunoglobulins and these can be obtained by experimentally inducing plasma cell tumors in some strains of genetically defined inbred mice. In addition, many of these myeloma proteins have been found to have antibody activity. Immunoglobulin determinants on both the common and variable regions of the heavy chain as well as conformational determinants involved in the antibody site provide tools to study the genetic control of the variability of antibodies. The finding of 5 identical myeloma proteins appears to indicate repetition of genetic mechanisms that control diversity of antibody production.

Project Subtitle II: Study of the Genetic Regulation of Antibody Synthesis

Project Description:

Objectives:

A. Determine the immune response to homologous genetic antigens (γA and γG myeloma proteins) in inbred and congenic strains with various combinations of immunoglobulin (Ig), histocompatibility (H_2) and complement component (C5-MUB1) genes.

B. Determine the immunoglobulin heavy chain linkage groups of random bred mice that have been bred and progressively separated on the basis of their high and low immune response to multideterminant antigens for many generations. Do selective backcross breeding and test progeny to determine whether there is linkage between genes controlling the immunoglobulin heavy chain and genes regulating antibody synthesis.

Methods Employed:

A. 1. Immunization of inbred and congenic strains of mice with various combinations of Ig, H_2 and C_5 (MUB1) genes with identical preparations of BALB/c IgA and IgG myeloma proteins.

2. Determination of antibody response based on production of anti-allotype and anti-idiotype precipitating antiserum.

3. Determination of the specificities of each antiserum produced.

B. 1. Determination of immunoglobulin phenotypes of low and high responders alternately immunized to sheep and pigeon erythrocytes after 16 generations of selection.

2. Testing of progeny of the appropriate backcross of low and high responders for immunoglobulin genetic markers to determine linkage or independent segregation of genes controlling the synthesis of immunoglobulin heavy chain and the antibody response.

Major Findings:

A. Mice from 30 different strains were immunized with one or more IgA myeloma proteins from BALB/c mice. Two types of precipitating antibodies were obtained; the more common form was antibody to idiotypic (Fab) specificities, the other was to allotypic (C_H) specificities. Neither type of antibody was obtained in mice that have the same C_H allotype as BALB/c mice. In strains of different C_H allotypic constitution, mice having IgA^{13} or IgA^{14}, H^{2a} or H^{2k} and lacking C_5 (MUB1) genes were generally the best responders; mice having IgA^{15} or IgA^- and H^{2b} or H^{2d} genes were poorer responders.

The immune response of congenic strains in the limited data thus far available, suggests that the H^2 and Ig C_H genes are linked to the antibody response. This is now being tested by the appropriate genetic crosses.

B. Two different immunoglobulin phenotypes were found after 16 generations of selective breeding of random bred mice for high and low response (agglutinin titer) to sheep and pigeon erythrocytes.

There were segregation of genes controlling the immunoglobulin heavy chains, suggesting linkage of the Ig C_H genes with those regulating antibody synthesis. The high responders were homozygous or heterozygous for two different heavy chain linkage groups (1/1, 1/2 or 2/2). The low responders were homozygous for one heavy chain linkage group (35/35). Appropriate backcross genetic tests are now in progress to establish the validity of these findings.

Significance to Biomedical Research and the Program of the Institute:

The immune response is a complex process involving many genes and in the mouse so far two different genetic systems have been implicated, the Ig and the H_2 and possibly a third, the C_5 (MUB1). In view of the wide variety of inbred strains, the mouse is by far the best animal to do studies involving the genetic control of antibody diversity with well defined antigens.

Proposed Course of Project:

To continue as outlined.

Project Subtitle III: The Role of Haptenic Groups in Supporting Immune Responses

Project Description:

Objectives:

The study of hapten-carrier relationships has led to many insights in immunology. The phenomenon of "carrier specificity" in eliciting anti-hapten immune responses, and the lack of such specificity in anti-hapten antibodies led to the discovery of cellular cooperation in the immune response.

The precursors of antibody forming cells are believed to bear immunoglobulin receptors on their surfaces and these receptors, in turn, appear to be identical to the product produced by the progeny of the precursor cells. A population of specific lymphoid cells which neither secretes detectable amounts of immunoglobulin nor differentiates into antibody secreting cells exist. The cells play a crucial role in the activation of precursors of antibody forming cells and are consequently termed "helpers". The receptors of these "helper cells" have yet to be characterized. However, the very fact of "carrier specificity" makes it likely that they differ in some way from conventional antibody.

The objectives of this project are twofold:

1) Can haptens function in a manner analogous to carrier specific immunity in the in the potentiation of antibody responses?

2) In our system, autologous anti-idiotype antibody is produced; - does this antibody detect idiotypic specificities in normal serum?

Methods Employed:

A mouse γF myeloma protein (MOPC 31C), arising in BALB/c mice, was conjugated with 2,4-dinitrophenyl (DNP) groups and injected into BALB/c and other inbred strains of mice some of which had been pre-iimunized to DNP by skin painting with fluorodinotrobenzene or by injecting DNP-protein conjugates. Antibody reacting with the Fab fragment of MOPC 31C - and anti-DNP antibody were measured by a highly sensitive micro-Farr technique.

An inhibition assay for MOPC 31C idiotype was developed, employing BALB/c anti-MOPC 31C and MOPC 31C-Fab labeled with iodine-125. Separation by sucrose density gradient permitted detection of MOPC 31C at 5-15 nanograms per ml.

Major Findings:

1) A series of experiments has shown that anti-hapten preimmunization suppressed or did not affect the anti-idiotype response to DNP-MOPC 31C. This effect was not due to circulating anti-DNP antibody, as infusion of anti-DNP antibody did not affect the responses to DNP-MOPC 31C. This finding is in accord with expectations that the receptors of "helper cells" differ from those of the precursors of antibody forming cells and from the binding sites of circulating antibody.

2) A search for MOPC 31C idiotype in normal sera is currently in progress. To date, no sera have been found with definite levels of this idiotype.

Significance to Biomedical Research and the Program of the Institute:

The cells that function as "helper cells' seem to play an important role in all immune responses, especially in recognition of antigens as foreign and in the induction of immune responses. An understanding of the specificity of their receptors is therefore important in understanding the immune response. If the specificity is different from conventional antibody, then is the receptor also different?

Proposed Course of Project:

1) Further DNP-myeloma proteins will be tested in this system to attempt to show that haptens can function as carriers.

2) As sera become available, idiotype will be sought and, if present, measured in normal sera.

Publications:

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ROCKY MOUNTAIN LABORATORY

HAMILTON, MONTANA

July 1, 1970 through June 30, 1971

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PHS-NIH

Summary Statement

Rocky Mountain Laboratory, NIAID

Hamilton, Montana

July 1, 1970 through June 30, 1971

The direction and support of our research program have been generally consistent with our defined mission, with primary emphasis on slow viral diseases, rickettsiology, medical entomology and acarology, and the immunology of tuberculosis, in particular. Reasonable scientific progress was achieved despite the net loss of 4 active scientists—Drs. C. B. Philip, Connie Clark, Jon A. Rudbach, and Alan Haakenstad. Compensation for part of this loss was realized through collaborative research efforts with 69 scientists in outside institutions. During the past year, we hosted 13 foreign and 29 native guest-worker scientists and visitors. Our active guest worker program and extensive collaboration with outside investigators reflect excellent peer recognition and interest in our research program, as highlighted in the following sections. Many of these findings have been reported in 55 publications.

Rickettsioses. During further inquiry on the role of ticks in the epidemiology of epidemic typhus, Dr. Burgdorfer examined 1,600 ticks (4 species) from Egypt and Ethiopia, and demonstrated spotted-fever-group rickettsias in 17 ticks. One Amblyomma cohaerens contained an organism that reacted specifically with Rickettsia prowazeki fluorescent antibody but all attempts to isolate the agent failed. Though previous attempts to infect adult African ticks with R. prowazeki failed, some larval Hyalomma dromedarii, Rhipicephalus simus, R. evertsi, and Amblyomma variegatum acquired mild infections after feeding on rickettsemic Microtus or guinea pigs, but none retained the organism to the adult stage. In view of the resistance of cattle and their ticks to infection with R. prowazeki, neither appear to play any role in the epidemiology of epidemic typhus.

Some recent serologic findings indicate that R. canada is pathogenic for man, but the source of infection and the ecology of the organism remain unsolved. Neither Dermacentor andersoni nor D. variabilis is a likely vector because the organism is highly pathogenic for these ticks. The lone star tick, A. americanum, appears to be a potential vector. Larval ticks infected on rickettsemic Microtus maintained infection transstadially and transovarially.

Using chick embryo primary cell cultures, Drs. Ormsbee and Wike have defined the optimum conditions for consistent production of plaques by the spotted fever and typhus group rickettsias. The former produce plaques 2.0 mm in diameter after 9 to 10 days incubation at 32°C whereas the typhus group produce smaller plaques (1.0 mm) after 12-13 days incubation. R. canada is unique because its plaque titer is higher than its infectious titer for eggs, mice, or guinea pigs. The ability to clone these organisms now permits studies of strain variations and genetic stability of pathogenic rickettsias.

Last year's studies by Drs. E. J. Bell and Munoz indicated that the R. typhi toxin-neutralizing (TN) factor in most normal human serum was not an immunoglobulin, but likely an alpha$_2$ macroglobulin. Although closely associated electrophoretically with this fraction, the TN factor has now been separated by DEAE column chromatography. Alpha$_1$ lipoprotein was separated from the TN factor by disc electrophoresis in polyacrylamide gel under conditions favorable for the rapid migration of alpha$_1$ lipoprotein. TN activity, after centrifigation of active serum in high density salt solutions, was found in the top layers that also contained beta lipoproteins and others of low density. Like beta lipoprotein, the TN factor was precipitated by dextran sulfate. Rabbit antiserum against dextran-precipitated TN-positive serum fractions neutralized TN activity of control positive human serum, whereas that produced against similar fractions derived from TN-negative serums did not do so. The TN factor, which now appears to be a beta lipoprotein or a closely associated fraction, should be better understood because it represents a nonspecific defense mechanism and may affect the interpretation of rickettsial serology.

Tick-borne viruses and western equine encephalitis (WEE). Considerable interest in tick-borne viruses was stimulated when the group B virus isolated last year from Ixodes uriae associated with seabird nests on islands near Tillamook, Oregon, was shown by Drs. Clifford, Thomas, and Yunker to be identical to Tuleniy virus, an agent recently recovered by Dr. Lvov from the same tick species collected on islands off the eastern coast of Russia. Large numbers of I. uriae were found this year on a Yaquina head near Newport, Oregon. Viruses were demonstrated in at least 40 of 100 pools (10 ticks each) tested in suckling mice and Vero cells. Some isolates were Tuleniy virus, and others appear to be related to the Kemerovo-Chenuda group viruses. Results of preliminary typing suggest that the 40 isolates belong to at least 4 different serological groups. It appears that seabirds and their ticks are extensively involved in the maintenance and dissemination of these viruses throughout the world.

Investigations started last year by Dr. Thomas on the occurrence and persistence of WEE viremia in garter snakes held at various temperatures have been completed. In previous work local snakes regularly developed viremia, but only 50% of snakes from Utah used in the present study developed viremia. These snakes were pretested for viremia and antibodies to WEE, but the antibody response to a vigorous challenge is so low that one questions the use of hemagglutination-inhibition (HAI) or neutralization tests for determining past exposure of snakes to WEE. Some of the snakes in the current study were re-inoculated with WEE virus 2 or [illegible] times; viremia did not recur, regardless of the presence or absence of demonstrable antibody. It appears that the controversial role of snakes in overwintering of WEE virus has not been clarified.

Biology of microbial agents in arthropods. Since the ovarian tissue of ticks, in particular, supports growth of many rickettsial and bacterial pathogens, Drs. Brinton and Burgdorfer have defined the developmental morphology, histology and fine structure of this organ as a base-line for interpreting potential tissue-parasite interactions. Interstitial cells, surrounding oogonia in unfed D. andersoni nymphs, become segregated in the adult as an epithelium lining the ovarial lumen. Oocytes rapidly develop while attached to funicular cells that have differentiated from the epithelial lining. When mature, oocytes pass through funical cells into the ovarial lumen. Maturation of oocytes was characterized by heavy emission of ribonucleoprotein from the nucleus, coalescing of ribosomes with vesicles budded as saccules from dictyosomal cisternae to form dense vesiculate bodies, and by morphogenesis of mitochondria to form membrane-limited multivesiculate bodies. By micropinocytosis, oocytes ingest hemocelic fluid which passes via small tubes to membrane-limited amorphous reservoirs in the ooplasm, which later fuse with dense bodies of intrinsic origin to form proteid-yolk granules.

Vector-pathogen relationships. In further study on this subject, Dr. Yunker has tested the ability of 18 arboviruses to propagate in a newly established line of cells from Culex quinquefaciatus and in primary cultures of tick hemocytes. In general, only culicine-borne viruses grew in mosquito cell culture and only tick-borne viruses grew in hemocyte cultures. With a new method of preparing hemocyte cultures, these cells have remained viable and supported growth of Colorado tick fever virus for 74 days. Hemocyte cultures would not support growth of all tick-borne viruses capable of growth in cultures of whole tick viscera. Rickettsias, unlike arboviruses, were not vector-tissue specific. Most rickettsias, including Rickettsia tsutsugamushi, grew well in mosquito cell lines. Data being accumulated on the growth of pathogens in arthropod cell cultures suggest that this system may be valuable for classifying viruses and studying their vector-pathogen relationships.

Systematics of arthropods. During the past year, Drs. Clifford and Keirans have enhanced the value of the RML tick collection through the continued transfer of the Hoogstraal collection and the recent acquisition of the tick collection of the Smithsonian Institution. Research has been chiefly directed toward a revision of Ixodes subgenera, identification of immature stages of Ixodes in the Western Hemisphere, and ticks of Venezuela and Nepal. Three new species were described this year.

Drs. Brennan and Yunker have been concerned chiefly with systematics of parasitic mites and chiggers, respectively, of South America. About 25 new species have been tentatively identified among 20,000 chigger specimens from Venezuela and new host records were established. Twenty-one new species have been recognized among samples of 800 collections of dermanyssoid mites from that country also. Descriptions of 15 new species, involving 6 genera, were published.

Zoonoses of regional importance. In continued research on abortive rabies, Dr. J. F. Bell has extended his observation on this phenomenon in dogs. In one experiment 3 of 5 dogs that developed paresis and other signs of rabies survived. The neutralizing antibody titers of spinal fluid of survivors were comparable to those in their serum, and titers remained high for 4 [illegible] months, the longest period tested. The absence of high antibody titers of spinal fluids of 550 dogs studied in a rabies-endemic area in Argentina attests to the specificity of the test. By simply testing spinal fluids of dogs, one could survey large populations in endemic areas to determine the prevalence of abortive rabies in natural infections.

In further studies on the immunology of abortive rabies Dr. Bell injected glass powder (anti-macrophage) or antithymocyte serum to alter resistance of mice to rabies. Both caused increased morbidity, but neither disturbed the survival rates. By inhibiting and stimulating immune and other curative systems, attempts are being made to elucidate the mechanism of abortive rabies infection.

Through our service, in part, as a regional diagnostic center for zoonoses, Dr. Owen made several interesting observations. Tularemia was shown to be a serious public health hazard to tourists in the recreational area controlled by the Tennessee Valley Authority. The organism was recovered from ticks and 30% of wild mammals tested had antibodies. A fatal human case of plague in Oregon was diagnosed by Ascoli tests of formalin-fixed tissues.

Psittacosis-lymphogranuloma-trachoma group. During the past year Dr. Philip has chiefly studied the antigenic interrelationships among chlamydial isolates from the Crow-Cheyenne Indian Reservation and from the District of Columbia. Using the microimmunofluorescence (micro-IF) procedure of Dr. Wang, he determined that the Crow strains were clearly type Ba, similar to those isolated from Indians in the Southwest. The DC TRIC isolates were antigenically distinct from the 6 recognized serotypes and probably comprise a new serogroup, along with several other untypable strains. Two serotypes of LGV (I and II) are now recognized and these are antigenically related to the B-D-E complex of TRIC serotypes. The DC LGV strains tested so far and all fast-killing TRIC strains appear to be type II. The antigenic relationship between TRIC and LGV may have epidemiologic implications. Antiserums against Crow trachoma strains are broadly reactive against the genital organisms, TRIC type E and LGV II strains. Trachoma among American Indians is so mild and self-limiting that perpetuation by eye-to-eye transfer appears unlikely. Hence, the genital tract may be the true reservoir among Indians.

Last year Dr. Gerloff reported the presence of toxin in a number of strains of chlamydia. He has now confirmed toxin in 13 chlamydial strains representative of both species. Toxin was thermolabile, could be inactivated by antiserum, but was not affected by doses of penicillin or sulfadiazine sufficient to interfere with inclusion development. Ancillary to this study, Dr. Gerloff devised a simple accurate procedure which permits calculating the number of particles per infectious or toxic dose.

Molecular biology of endotoxins. A variety of research projects on endotoxin (ET) were undertaken, but tolerance to ET, metabolism of ET, and structural organization of ET were chiefly studied.

Research by Drs. Milner and Rudbach was designed to establish the true nature of the transfer factor effective in late tolerance; they showed that tolerance in rabbits and mice could be mediated by O antibody alone. Immunization with ET from O antigen deficient mutants produced little transfer factor to homologous or wild-type ET, whereas immunization with wild-type ET was highly effective in both respects. Late tolerance and passive transfer were specific, even among related organisms such as colon bacilli and salmonellae. Early tolerance, in contrast, shows no interendotoxic specificity and passive transfer has been impossible with nonpyrogenic sera drawn within 24 hours of a single dose of ET.

In a collaborative project with Mrs. Jackson, Dr. Rudbach studied the distribution and retention of radiolabeled endotoxin after mice were inoculated intravenously with graded sublethal doses. During the 12 weeks of the study, 60 to 90% of the inoculated radioactive marker was retained, in decreasing order, in the liver, spleen, lungs, and mesenteric lymph nodes. Adjustments of doses between submicrogram and overtly toxic amounts did not affect its distribution or persistence. These findings suggest that ET is not simply excreted and that its long persistence may help explain the unusual "immunologic memory" to molecular amounts of lipopolysaccharide (LPS) antigens.

The structural organization of bacterial endotoxin, as it may relate to its mode of action, has been of primary interest to Dr. Ribi. This year he compared, by electron microscopy, the structural elements seen in elaborately refined highly toxic endotoxin with those seen in nontoxic lipopolysaccharides, e.g., those obtained from Francisella tularensis. When dried films of toxic preparations were stained negatively with potassium phosphotungstate, he observed discs of about 500 Å in diameter, free of detailed fine structure, and ribbon-like structures about 200 Å wide, whose length varied from short rectangles to long branching filaments. Sometimes, rectangular particles and ribbons contained an unstained core surrounded by stained areas that could be interpreted as deposits of stain between fine side chain branches. Essentially similar structures of the same size and shape were seen in lipopolysaccharide preparations of Francisella tularensis treated and examined the same way. These studies suggest that submicroscopic morphology of endotoxin can not lead directly to conclusions about its mode of action.

Centrifugal chromatography in silica gels. This procedure, as developed by Dr. Ribi in collaboration with Ivan Sorvall, Inc., has been found useful for separating a wide variety of substances, including components of mycobacterial Wax D, sugars, lipids, and steroid hormones. The resolution that can be obtained by this procedure is sufficient to separate chemical isomers from each other. This year the procedure was used for separating, identifying, and quantitating the cannabinoid components of marihuana and hashish. Cannabichromene, cannabinol, Δ^9-tetrahydrocannabinol, Δ-tetrahydrocannabinol, cannabidiol, and cannabicyclol were all separated from each other. The migration rates (ratio of distance migrated to length of tube) were 0.11, 0.22, 0.52, 0.60, 0.76, and 0.91 respectively, and were reproducible within 0.01. The chromatograms were sprayed with di-o-anisidine tetrazolium chloride reagent to produce specific color reactions. By comparing width and color density of bands with known quantities of synthetic standards, the amounts of major cannabinoids in street samples of these drugs could be determined. Ten materials commonly used to dilute marihuana did not contain substances which could interfere with analysis. This technique meets the needs of both the chemist and physiologist involved in research on marihuana and overcomes the difficulties associated with separating cannabinoids by thin-layer and gas-liquid chromatography.

Immunology of tuberculosis. In our research on tuberculosis, the immunology of living BCG vaccine in monkeys and the relationship between delayed hypersensitivity and immunity received principal attention.

Previously Drs. Anacker and Ribi showed that BCG cell-wall vaccine in monkeys was more effective when given intravenously than by any other route. In a further collaborative study with the Naval Biological Laboratory they showed that the potency of living BCG vaccine also was dependent on route of administration. Protection in vaccinated monkeys autopsied 10 weeks after airborne exposure to virulent bacilli was numerically graded according to the extent of disease; those with no lesions were scored 0 and those with maximal disease were scored 100. Mean scores for controls were 63, 0 for intravenously vaccinated (1 mg), [illegible] for subcutaneously vaccinated (0.1 or 1 mg), and 38 for intramuscularly vaccinated (1 mg). These scores are roughly comparable to those obtained before with cell-wall vaccine.

Living BCG vaccine, like cell-wall vaccine, produced adverse reactions. Those given 1 mg BCG subcutaneously developed nodules that later abscessed and drained. Those given vaccine intravenously lost weight temporarily and had minor but definite histologic pulmonary changes. However, they did not develop the extensive granulomatous pulmonary changes seen in monkeys given cell-wall vaccine intravenously.

The role of delayed hypersensitivity in tuberculoimmunity has long been a controversy and some contend that the two are correlated. However, observations by Drs. Anacker and Ribi did not support this thesis. Monkeys given living BCG intravenously and intramuscularly had comparably high levels of sensitivity to PPD; the former were fully protected against airborne challenge, whereas the latter were, at best, only partially protected. When mice were given nonviable vaccines of varying potency and a live BCG booster dose several weeks later to accelerate development of delayed hypersensitivity, few mice developed any reaction to the footpad test given several days after the BCG dose; no correlation between delayed hypersensitivity and resistance could be demonstrated.

Studies of the components of Wax D and other lipid fractions have been continued by Drs. Smith and Ribi with the aid of centrifugal microparticulate-bed chromatography. With equipment available it has been possible to separate Wax D, for example, into 7 or more fractions, but only submilligram quantities can be processed at one time. Hence, it has been impossible to obtain adequate samples of pure fractions for potency tests in mice. Some success has been achieved by fractionating samples up to 50 mg in size through relatively large beds of microparticulate gel under the influence of pressure rather than centrifugal force or through large columns of glass beads with highly porous surfaces.

Bordetella pertussis antigens. As previously reported, Dr. Munoz demonstrated a reaginic antibody in mice given hen egg albumin mixed with saline extract of Bordetella pertussis (HSF). Very small amounts were produced and, as in the case of human nonmyeloma γE, purification has been difficult. Relatively pure fractions obtained by disc electrophoresis still contained γ_1-γ_2 globulins. Serum from guinea pigs immunized with these fractions was rendered specific by absorption with whole mouse serum. The absorbed antiserum failed to react with all known immunoglobulins of mice (IgG_1, IgG_2, IgM, IgA). It had distinct heavy chain specificity and L chains similar to IgG globulins. Based on its properties and concentration in normal serum, it appears to belong to a class similar to γE of man.

Slow viral diseases. Continued emphasis has been placed on the pathogenesis of 4 slow viral diseases—scrapie and progressive pneumonia of sheep, and Aleutian disease and encephalopathy of mink. Experiments begun several years ago by Dr. Hadlow have now yielded results which provide more insight into the relationship between scrapie and mink encephalopathy (ME) viruses. These viruses have common attributes and can be distinguished only by experimental host susceptibility. Pastel mink that were inoculated intraperitoneally with scrapie virus and remained normal for 4 1/2 years were challenged intracerebrally with 10^4 LD_{50} of ME virus. All developed typical encephalopathy 6 to 7 months later. Thus scrapie virus failed to cause disease or to protect them against ME. We have found that ME does not cause disease in mice and others have reported goats to be similarly refractive. However, recently, 7 of 19 goats inoculated with Idaho ME virus about 3 years ago have developed a progressive neurologic disease resembling scrapie. The clinical signs and histopathologic changes were similar to scrapie except for a fairly consistent spongiform change in the cerebral cortex. Hence, further comparative studies will have to be made before the true relationship between scrapie and ME viruses can be established.

Drs. Hadlow and Race have extended their investigations of the pathogenesis of Aleutian disease (AD) in pastel mink, a color phase less susceptible than sapphires. Of 20 inoculated subcutaneously with 2 X 10^4 LD_{50} AD virus, about half had viremia at 2 and 4 weeks, but only 5 continued [illegible] One died from AD at 10 weeks and the other 4 have remained normal through 24 weeks, though their serum gamma globulin levels were moderately elevated. Six mink never had viremia or elevated gamma globulin levels. These studies indicate that the pathogenesis of AD, like progressive pneumonia and possibly other slow viral infections, is influenced by the genetic pattern of the host.

Virologic studies by Dr. Lavelle were oriented chiefly toward finding ways of cultivating slow viral agents in vitro. An attempt was made to separate subpopulations of scrapie-virus-rich cells from spleens of mice known to harbor high concentrations of virus. By differential flotation of spleen cell suspensions in density gradients of isotonic albumin, it was possible to obtain cell populations from levels with lower specific gravity that contained 50- to 100-fold more virus than the whole suspension. This technique may be of value in identifying the type of cell capable of supporting virus growth.

Continued efforts to demonstrate AD virus in tissue culture have been unsuccessful. Cytopathic effects or other changes have not been seen in primary explants of virus-bearing tissues or in a variety of mink cell monolayers inoculated with AD virus. In other experiments, cultures of splenic macrophages and of other spleen cells have been maintained for 2 weeks; survival of these cells from AD-infected mink was comparable to that of controls.

Allergy and Immunology. Research efforts this year were directed chiefly toward studies of the immune mechanisms of normal mink and the changes induced by Aleutian disease (AD) on antibody synthesis and the reticuloendothelial system. Besides the IgG and IgM previously described, an IgA has now been characterized by Dr. Coe. It had L chain determinants in common with other Ig, but had unique heavy chains. Preliminary observations suggest marked increase of this Ig in AD-affected mink. All mink (male and female, sapphire and pastel) were found to have 2 antigenically distinct L chains, of which chain "a" had unique determinants.

Drs. Lodmell, Bergman, Hadlow, and Race studied the effect of AD on antibody synthesis to goat erythrocytes (G-RBC) in pastel and sapphire mink inoculated with AD virus 1 day after or 47 days before primary immunization. Pastel and sapphire mink given AD virus 1 day after antigen (G-RBC) had comparable numbers of 19S and 7S plaque forming cells (PFC) in their spleens and lymph nodes at various times after immunization. However, after a booster dose (G-RBC), peak 7S and 19S responses were reached earlier in sapphires, but the hemolysin titers were slightly higher in pastel mink. Of [illegible] mink of each color phase that became AD-affected, the pastels had more PFC and higher hemolysin titers.

Response to G-RBC was markedly different in mink inoculated with AD virus 47 days before antigenic stimulation. When G-RBC were given, all sapphires but only 9 of 20 pastels were affected with AD. Neither primary nor anamnestic 19S or 7S PFC were detected in spleens of AD-affected sapphires, but some primary and secondary responses were noted in cells from abdominal lymph nodes (ALN). In contrast, both AD-affected and unaffected pastel mink had considerable numbers of 19S and 7S splenic PFC, but more were detected in unaffected mink. All pastel mink, regardless of their clinical condition, had primary and anamnestic 19S and 7S PFC in ALN. Whereas unaffected pastels had the greatest number of PFC in ALN, AD-affected pastels were more responsive than AD-affected sapphires. The peripheral lymph nodes of unaffected or AD-affected mink did not appear to be significantly involved in the antibody response.

The impairment of the reticuloendothelial system induced by AD was evaluated by comparing the clearance of ^{125}I-microaggregated human serum albumin (^{125}I-HSA) and colloidal carbon from the blood stream of normal and AD-affected mink. Normal mink and those with early clinical AD removed comparable amounts of ^{125}I-HSA from the blood stream, but more was retained in the spleen, liver, and bone marrow of normal mink than in those with early AD. In contrast to findings with ^{125}I-HSA, colloidal carbon was removed faster from the bloodstream of normal mink. AD-affected mink in advanced disease had a reduced capacity to clear both ^{125}I-HSA and colloidal carbon.

The foregoing findings provide some insight into the pathogenesis of AD and offer one explanation for the greater susceptibility of sapphire mink. The mere presence of large amounts of AD virus does interfere with antibody production, but once the disease is well-established, the ability to respond to new antigens is drastically altered.

In continued studies of delayed hypersensitivity in guinea pigs to bovine insulin and its A and B polypeptide chain components, Dr. Munoz used the macrophage migration inhibition test to study the specificity of these reactions. Macrophages from insulin or DNP-insulin sensitized animals were inhibited by insulin, B chain, and A chain. Macrophages from animals desensitized with insulin migrated in the presence of insulin and B chain. It was interesting to note that neither changes in glucose metabolism nor histopathologic changes were seen in guinea pigs highly sensitive to bovine insulin.

Serial No. NIAID-170
1. Rocky Mountain Laboratory
2. Office of the Director
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Office of the Director

Previous Serial Number: Same

Principal Investigator: H. G. Stoenner, Director

Other Investigator: R. N. Philip, Assistant Director

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	48			48
Professional:	1			1
Other:	47			47

Project Description:

Though research staff have considerable freedom in the conduct of their own research program, the director is responsible for general direction of programs and changes in emphasis as needed to meet research goals. Considerable progress was made the past year in involving staff of the Allergy and Immunology Section in research on problems in slow viral diseases and rickettsiology.

This office is also responsible for allotment of space and facilities, assignment of technical personnel, and general research support services such as clerical assistance, animal production, library and graphic arts services, general administration, and general maintenance of buildings and grounds. However, personnel engaged in more direct support, such as glassware and media preparation and care of animals under experimentation, are allotted to each project in proportion to support given.

Honors and Awards:

Dr. H. G. Stoenner

Continued as a Reviewer for American Journal of Veterinary Research.

Continued service on the Committee on Environmental Health of the Montana State Advisory Council on Comprehensive Health Planning.

Continued service on the Board of Directors of the Montana Chapter of the American Cancer Society.

By invitation, presented a paper on Differential Diagnosis of Leptospirosis at a Symposium on Viral Diseases sponsored by CDC, the Idaho Medical Association and the Idaho Veterinary Medical Association, Boise, Idaho, October 1970.

Awarded DHEW Distinguished Service Medal, April 1971.

Serial No. NIAID-171
1. Rocky Mountain Laboratory
2. Rickettsial Diseases Section
Serological Technology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Rickettsiae and rickettsial infections

Previous Serial Number: Same

Principal Investigators: E. J. Bell, W. Burgdorfer, and R. A. Ormsbee

Other Investigators: L. P. Brinton, C. M. Clifford, R. K. Gerloff, L. E. Hughes, J. J. Munoz, M. G. Peacock, L. A. Thomas, D. A. Wike, and C. E. Yunker

Cooperating Units: Dr. J. C. Cooney, Tennessee Valley Authority, Muscle Shoals, Alabama
Dr. K. Johnson, MARU, Canal Zone
NAMRU-3, Cairo, Egypt
University of Maryland School of Medicine, Baltimore: Dr. P. Fiset and Dr. C. L. Wisseman, Jr.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	11 3/12		2/12	11 5/12
Professional:	5		2/12	5 2/12
Other:	6 3/12			6 3/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
03-008-1 (PL-480)	W. Burgdorfer	NAMRU-3 Cairo, Egypt	H. Hoogstraal	$31,002

Project Description:

Objectives:

1) Continued investigations on the role of domestic and wild animals and of ticks in the natural history of epidemic typhus in Africa, 2) development and improvement of serologic and microbiologic methods useful in research on rickettsial diseases, 3) development of improved diagnostic reagents and rickettsial vaccines, both killed and attenuated, 4) studies of prevalence and epidemiology of rickettsioses in selected areas of the world, 5) antigenic analyses of natural groups of pathogenic rickettsiae, i.e., spotted fever group, typhus fever group, Q fever group, 6) development of tissue culture techniques which will enable studies on genetic characteristics and strain variation to be effectively prosecuted, and 7) attempts to isolate and identify a nonantibody factor in normal human serum which neutralizes the toxic effect of Rickettsia typhi.

Methods employed:

Standard methods in rickettsiology have been employed. This means standard animal tests with guinea pigs, mice and embryonated eggs and standard agglutination and complement fixation tests. In addition, a variety of tissue culture systems have been employed, and a number of standard chromatographic, electrophoretic and immunologic techniques for the separation and characterization of serum components.

Major findings:

1. Typhus toxin-neutralizing (TN) factor in human serum. The typhus TN factor which selectively neutralizes the toxic action of R. typhi was totally excluded by Sephadex G-200 in a column along with other high molecular weight serum proteins. Although closely associated electrophoretically with α_2 macroglobulin, it could be separated from the latter by DEAE column chromatogrphy in which the salt concentration was varied during elution. Precipitation of the TN factor, but not α_2 macroglobulin, by dextran sulfate or heparin, has been demonstrated.

Evidence that the TN factor is not related to IgM has been obtained from serum absorption tests, ultracentrifugation and filtration in a Sepharose column. α_1 lipoprotein was separated from the TN factor by disc electrophoresis in polyacrylamide gel under conditions in which α_1 lipoprotein, but not TN factor, migrated rapidly.

TN activity, following centrifugation of active serum in high density salt solutions, was found in the top layers, which also contained β lipoproteins as well as other lipoproteins of low density. About 75% of the TN activity of positive sera could be precipitated by dextran sulfate; thus it behaved like β lipoprotein. Rabbit antisera against dextran-precipitated TN-positive fractions from human positive sera were capable of neutralizing the TN activity of control positive human sera. However, corresponding antisera against the dextran-precipitated fractions derived from TN negative human sera did not neutralize the TN activity in control positive human sera.

2. Rickettsioses in Central America. A survey of 2,000 Central American sera was undertaken, not only in a retrospective search for rickettsioses but also to evaluate new procedures and reagents.

The first evidence for rickettsialpox in this region was found in sera from Costa Rica. In addition, serologic evidence highly suggestive of R. canada infection was found in Guatemalan sera. Evidence of past epidemics of epidemic typhus correlated well with known historical records of this disease in the Guatemalan highlands. Both the CF and the microagglutination (MA) tests (with washed, purified particulate antigens) were found useful in this survey. The MA test was highly specific particularly in identifying antibodies against various members of the spotted fever group.

3. Tick-borne rickettsioses in Ethiopia and Egypt. A total of 1,607 live ticks including 1,213 from Ethiopia and 394 from Egypt, of several species and from different domestic animals, have been examined for rickettsial infection by the hemolymph test and with fluorescent antibody (FA) staining. Spotted fever group infections were found in 47 ticks (11 Amblyomma variegatum, 17 A. cohaerens, 19 A. gemma) from the Ethiopian High Plateau. One spotted fever group isolation was made from progeny of a single Hyalomma dromedarii collected in Egypt. One A. cohaerens collected in Ethiopia contained a rickettsia-like agent which appeared to react specifically by FA with antisera prepared against R. prowazeki. All attempts to isolate it from the tick failed.

4. Growth of rickettsiae in tissue culture. Continued efforts have been made to define optimum conditions for plaque formation in chick embryo primary cell cultures. We now have produced plaques reliably with R. rickettsi, R. conori, R. typhi, R. prowazeki, and R. canada. Coxiella burneti alone has been less than satisfactory in plaque production in primary chick embryo cells. Spotted fever group rickettsiae produce plaques 2.0 mm in diameter after 9 to 10 days incubation at 32°C; typhus group rickettsiae produce smaller plaques (1.0 mm in diameter) and take longer to develop (1[illegible]-1[illegible] days at [illegible]°C). R. canada is unique among pathogenic rickettsiae because its plaque-forming titer is higher than its infectious titer by any other method tried, including embryonated eggs, mice, and guinea pigs.

Plaque isolation techniques have been developed and plaque isolates have been made, preparatory to studies of strain variation and genetic stability in pathogenic rickettsiae.

R. sibirica has been examined in conventional tissue culture in 5 lines of heteroploid human or mouse origin. All cell types have supported luxuriant growth of R. sibirica and appear superior to primary chick embryo cells in this regard.

5. Phase variation in C. burneti. This subject continues to evade final answers. The mechanism of phase change (genetic or adaptive) is still unknown. Examination of 306GP/3EP Nine Mile C. burneti by an indirect ferritin-tagging technique indicated that a significant percentage of the organisms were in phase II, even though this particular strain had been passed only twice in embryonated eggs. Attempts are underway to make pure phase I antiserum with a DMSO extract of phase I rickettsiae as the immunizing agent, and to make pure phase II hyperimmune serum using the Australian QD strain as the immunogen.

6. WHO Reference Center function. As a WHO Regional Reference Center for Human Rickettsioses, RML last year supplied on request 8 strains of rickettsiae, 3 samples of C. burneti antigen and 7 samples of standard antiserum. In addition, but not as a WHO activity, RML deposited 7 strains of rickettsiae in the American Type Culture Collection. We currently have on hand for WHO reference purposes and as RML research reagents 12 strains of C. burneti, 7 of R. prowazeki, 2 of R. typhi, 3 of R. rickettsi, 2 of R. conori, 2 of R. sibirica and 1 each of R. akari and R. canada.

The Serology Unit has continued to participate in the CDC Proficiency Testing Program as a Referee Testing Laboratory. It has also continued to perform standard serologic tests for rickettsial antibodies on specimens submitted by physicians, veterinarians, hospitals, clinics, State Health Departments, foreign domestic investigators, and to exercise surveillance of RML personnel for serologic evidence of rickettsial disease. In addition, it has collaborated with RML investigators in performance of standard serologic tests.

7. In collaboration with the Tennessee Valley Authority, we undertook a study to evaluate the hazard of tick-borne diseases in a recreational area of 170,000 acres in northwest Tennessee and western Kentucky. Blood samples (288) and ticks (648) were obtained from a wide variety of fauna and tested for rickettsial and bacterial agents. Of 6 species of ticks tested, Dermacentor variabilis was most frequently infected with R. rickettsi. Among 13 species of animals from which blood samples were obtained, only cottontail rabbits had antibodies to R. rickettsi. Typhus group antibodies were detected in 1 raccoon, rabbit and feral dog.

[illegible]. See projects NIAID 186 and 188 for related research on rickettsioses.

Significance to bio-medical research and the program of the Institute:

Current information on a toxin-neutralizing factor found in sera from some individuals indicates that it is not an antibody but is closely associated with the β lipoprotein fraction of human serum. This material, which neutralizes the toxic effect in mice of *R. typhi*, is not present in sera from all individuals although a β lipoprotein component presumably is present in all normal human sera. This substance as well as those substances found in sera of domestic animals, which may cause the nonspecific fixation of complement in the presence of rickettsial antigens, need to be better understood because they represent nonspecific body mechanisms for reacting with foreign antigen and, in addition, pose a problem in the interpretation of serologic results.

Current surveys of antibodies in Central America and of tick-borne rickettsioses in Africa have helped to give us a better current picture of the extent and nature of rickettsial disease in Central America and the intensity and distribution of tick-borne rickettsial infection in Egypt and Ethiopia and thus to predict with a sound basis the nature of human rickettsial disease which may occur in these areas. Evidence for *R. akari* and *R. canada* infections in Central America have been shown for the first time; the prevalence of spotted fever group infections in Ethiopian ticks and the lack of such in Egyptian ticks make it likely that this finding will be reflected in human rickettsial disease in these two countries; the lack of evidence for *R. prowazeki* in ticks in Ethiopia and Egypt, the failure to cause solid systemic infections of *R. prowazeki* in ticks and the continued difficulty encountered in trying to establish *R. prowazeki* infections in young sheep all suggest that there is no important extra-human cycle of epidemic typhus in Egypt and Ethiopia.

Experimentally, the development of a plaque technique for rickettsiae offers the obvious and attractive possibility of permitting establishment of clonal lines of rickettsiae for preparation of standard diagnostic materials, and a method to study genetic variation and variability in such areas as the phase I- phase II relationship in *C. burneti*, the antigenic relationships between spotted fever group and typhus group organisms, the relationships between *R. canada* and *R. prowazeki*. The successful growth of *R. sibirica* in mouse L cells suggests that other spotted fever group rickettsiae may be grown more successfully in tissue culture than in embryonated eggs to provide antigens and toxins useful for further investigation of the spotted fever group of rickettsiae.

Characteristics of phase variation and its control lie at the base of reliable vaccines and diagnostic reagents for Q fever. We still are not certain about the prophylactic significance of phase II antibodies, we still do not understand the apparent conservation of phase II antigens in nature or the mechanism of loss of phase I antigen in yolk sac culture. The whole subject of phase in C. burneti thus remains one of public health as well as fundamental biological importance.

Proposed course of project:

Research on the nature of the TN factor in normal human serum will continue. Attempts will be made to purify fractions of low-density lipoproteins and to determine the immunologic differences between lipoprotein fractions from the TN-positive and TN-negative donors.

Investigations will also continue on the antigens of R. canada and its relationship to the spotted fever and typhus groups of rickettsiae.

Tissue culture techniques will be utilized in efforts to examine antigenic heterogeneity and genetic stability in rickettsiae. This can be done perhaps to best advantage in the spotted fever group. Continued efforts will be made to characterize species of Rickettsia in terms of biological parameters such as relative infectiousness in different systems and relate this to the absolute rickettsial count. Studies will continue on the characterization of antibodies formed in experimental animals as a result of infection with R. prowazeki.

Continued efforts will be made to characterize soluble phase I antigen from C. burneti, to produce specific and pure phase I and phase II antigens and antibody preparations and to delineate more clearly the role of phase II antibodies and the natural biological relationship between populations of phase I and phase II organisms.

A retrospective survey of Egyptian rodent sera will be made with particular attention to spotted fever group antibodies.

Publications:

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Ormsbee, R. A., Peacock, M. G., Bell, E. J., and Burgdorfer, W.: Experimental infections of lambs with Rickettsia prowazeki. Amer. J. Trop. Med.

Fiset, P., Wike, D. A., Pickens, E. G., and Ormsbee, R.: Antigenic comparison of strains of Coxiella burneti. Acta Virol.

Serial No. NIAID-173
1. Rocky Mountain Laboratory
2. Zoonoses Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Selected zoonoses of regional importance

Previous Serial Number: Same

Principal Investigator: J. F. Bell

Other Investigators: W. Burgdorfer, B. M. Hestekin, Cora R. Owen, and L. A. Thomas

Cooperating Units: Dr. Myron A. Andrews, Dept. Veterinary Medicine, North Dakota State University, Fargo
Dr. J. C. Cooney, Tennessee Valley Authority, Muscle Shoals, Alabama
Dra. A. M. Diaz, Centro Panamericano de Zoonoses, Ramos Mejia, Prov. de Buenos Aires, Argentina
Dr. E. L. Hansen, Wildlife Research, Indiana Dept. of Natural Resources, Indianapolis
Dr. Robert Hoffman, Zoology Department, University of Kansas, Lawrence
Dr. W. L. Jellison, Parasitologist Emeritus, Rocky Mountain Laboratory, Hamilton
Dr. Philip Wright, Department of Zoology, University of Montana, Missoula

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	4 11/12			4 11/12
Professional:	3 8/12			3 8/12
Other:	1 3/12			1 3/12

Project Description:

Objectives:

Elucidation of the ecology of Francisella tularensis, rabies virus, and other zoonotic microorganisms and investigation of the pathogenesis and immunology of some zoonoses.

Methods employed:

Field work in ecology and routine laboratory techniques of microbiology, entomology and immunology are employed as necessary for surveillance of regional outbreaks of disease.

Major findings:

1. Epizootiology of tularemia. Monitoring of local streams for water-borne tularemia has been renewed and expanded with emphasis on attempts to localize and identify the source of contamination. Prevalence of infectivity in water samples has been low (14/216 = 6.5%). There is some evidence of attrition in bacterial populations downstream, as the source of contamination fades.

A project was undertaken with Dr. Cooney (TVA Authority) to evaluate public health hazards, especially from tularemia and Rocky Mountain spotted fever, in the Land Between the Lakes, a federally administered site in contiguous areas of Tennessee and Kentucky. Ticks (648) and mammalian serums (268) received from the area were tested for evidence of infection. F. tularensis was isolated from 2 Dermacentor variabilis and 1 Ixodes spp., and serologic evidence of infection was significantly high, especially among gray foxes, raccoons, opossums, and skunks. A total of 81 (nearly 30% of all animals tested) had agglutinins to F. tularensis in titers as high as 1:160.

Cooperative studies with Dr. Hansen on tularemia in penned cottontail rabbits (a hunting area) were continued with lessened activity. F. tularensis was isolated from 2 of 7 lots of I. cooleyi and 2 of 3 rabbit spleens.

The antigenic specificity and interrelationships of several organisms of the Pasteurella group (P. multocida, Yersinia pestis, Y. pseudotuberculosis, Y. philomiragia, F. tularensis and F. novicida) were investigated this year but with very little result, because our blood-enriched medium was inadvertently prepared with blood from animals fed antibiotics. These experiments are being repeated.

2. Rabies.

a. Rabies in skunks. In collaboration with Dr. Andrews, who is studying the epizoology of rabies in North Dakota, we are doing neutralization tests on serums and brains of skunks. Thus far, we have tested 120 serums and 43 brains. Forty-four were paired specimens (serum and brain from the same animal). Seven serums reacted in dilutions ranging from 10 to 270. Only 2 brains were available from animals with positive serums, but neither reacted at a 1:10 dilution. All other brains were likewise negative. It appears that none of the 44 animals with paired specimens had recovered from rabies, since all had negative brain neutralization tests. However, we do not have a satisfactory explanation for the high specific antibody titers in serum.

b. Abortive rabies in dogs. In experiments reported earlier, we described induction of abortive rabies in 2 dogs, recovery of the dogs from illness, and confirmation of infection with the brain neutralization test which had previously been found reliable in mice. Spinal fluids had neutralizing antibody titers comparable to those in the brain and blood. Subsequently we induced abortive infection with recovery from paresis in 3 of 5 affected dogs (the other 2 died of rabies). Spinal fluid titers remained high for at least 4 1/2 months after recovery, thus permitting unequivocal retrospective diagnosis for at least that length of time. The dogs are still alive and will be tested further. Dogs inoculated identically but without signs of infection had low antibody titers in both serum and spinal fluid.

c. Immunology of abortive rabies in mice. Glass powder administered to mice prior to injection of virus and antithymocyte serum given at the time of inoculation of virus significantly increased the incidence of illness and total mortality. However, there was not a proportionate decrease in the frequency of survival with sequellae in animals that became ill; this finding confirms the independence of the two phenomena. In the antithymocyte-treated mice, there was some indication of rebound effect which suggests that time relationships will be very important. We are currently attempting to elucidate the mechanisms of aborting infection by inhibiting and stimulating immune and other curative mechanisms.

d. Bat rabies. Of 111 bats examined for rabies in 1970, 2 were infected. This is the 17th successive year that rabies has been demonstrated in bats in this area. Although our surveillance of bat rabies is primarily service to local, state and federal agencies, we are interested in the fact that this disease continues to smoulder in enzootic form in the several species of bats without apparent spread to other mammals. Only 24 animals of other species were examined, but all of them were submitted because they were suspected of being infected, a selection procedure that greatly increases chances of finding infection. Failure of spread is more remarkable because we have repeatedly observed the aggressiveness of naturally infected bats and have often demonstrated transmission of rabies from naturally infected bats to laboratory animals. We have also shown transmission of rabies to striped skunks (but not cats) by ingestion.

3. This laboratory continues to serve as a diagnostic center for miscellaneous specimens submitted by RML staff, as well as veterinarians, physicians, game biologists, university staff and public health agencies.

The following findings are noteworthy:

a. A decline of the English sparrow population in western Montana, particularly in Missoula, has caused comment. Many sparrows were seen in [illegible] and acting in unusual manner. Group B _Salmonella_ was isolated from 3 sparrows submitted by Dr. Wright, Department of Zoology at the University of Montana.

b. _P_. _multocida_ was recovered from 6 sheep (from Dr. Hadlow), 6 bobcats, 1 lynx, 1 cougar and 1 feral cat.

c. An Oregon physician sought confirmation of tularemia in a rabbit hunter. However, organisms resembling _Yersinia_ sp. instead of _F_. _tularensis_ were seen in fixed and stained smears. Formalin-fixed tissues (the only tissues available) were requested and an Ascoli extract of those tissues was positive against commercial antiplague serum, but not against normal serum, anti-_Francisella_ or anti-_Pasteurella_ serums. We learned subsequently that _Y_. _pestis_ was isolated in Oregon from the patient's blood.

d. Published reports (Emmons et al.) of isolation of _F_. _tularensis_ from a pet squirrel monkey (_Saimiri_ _sciureus_) in California described several peculiarities of the organism, viz., anaerobic better than aerobic growth, good growth on tryptose blood agar and no growth in thyoglycolate medium. However, the organism as received and studied here did not exhibit these atypical characteristics, possibly because of our difficulties with culture media.

Significance to biomedical research and the program of the Institute:

As outdoor recreation continues to become more popular with the American public, new habitats are being invaded at all seasons. Inevitably this invasion results in greater contact with intercurrent diseases of wild animals which now pose threats to public health. Tick-borne spirochetosis in the Sierras, skunk rabies on the prairie, _F_. _tularensis_-infected streams, and plague in desert rabbits are increasing sources of infections among people who have only casual contacts with nature and do not take preventive precautions. When these infections occur, there is usually pressure (sometimes hysterical) to eliminate disease by eradication of hosts or vectors with pesticides. Often such campaigns do more harm than good. The best way to avoid ill-advised "control" measures is to know what is occurring. "For intelligent interference with the course of events, it is essential to know what is actually happening."

Monitoring of streams for many years has demonstrated _F_. _tularensis_ in some of them repeatedly. However, the source remains an enigma. Research is planned on procedures that we hope will shed some light on the nature of the source of contamination.

Tularemia is widespread and causes frequent illness in man. The organism has an extremely diversified ecology and is found in a variety of arthropod and vertebrate hosts as well as streams. Moreover, the disease is subject to great fluctuations in prevalence. Present knowledge is fragmentary and inadequate. Continued investigation should furnish information of value in diminishing occurrence of the disease in man. The same is true of other regional zoonoses, including rabies in bats. It is also true that we consider our efforts very inadequate because of lack of personnel and facilities in proportion to the enormity of the problems and the opportunities for profitable study.

Abortive rabies is being attacked on three fronts with differing emphases. Wildlife rabies is a greater problem than canine rabies in this country and the skunk is one of the 2 most common species affected. We cannot mount an independent study in Montana but are collaborating with Dr. Andrews to facilitate and broaden his good study of skunk rabies in North Dakota.

Our work on abortive rabies in dogs is intended primarily to evaluate the potential that dogs with nonfatal, unrecognized rabies are sources of disease to other animals and man. Although we were unable to detect abortive rabies in 550 dogs studied in a highly enzootic area in South America we have shown that abortive rabies can be experimentally produced in dogs. One outcome of these experiments is the demonstration that the cerebrospinal fluid neutralization test is reliable, rapid and inexpensive. It can be used to study larger numbers of animals with greater facility than heretofore possible.

The third aspect, and the one receiving greatest emphasis now, is the manipulation of immune and other mechanisms participating in curative processes. The effects of chronic, acute, specific and nonspecific inflammation in the central nervous system upon permeability of the blood/brain barrier are pertinent to this issue.

Proposed course of project:

We will continue to study the ecology of F. tularensis in local streams and in vertebrates and arthropods of this region, Indiana, and the TVA site. Specimens from the TVA will also be tested for certain viruses. Surveillance of rabies among bats, skunks and other animals will be continued. As in the past, miscellaneous referred specimens will be tested as a courtesy to federal, state, and local public health agencies, physicians, veterinarians and others and for the opportunities for surveillance that such testing affords.

Studies on pathogenesis of rabies and recovery mechanisms will be intensified because other studies, e.g., survey of pound dogs, have been dropped and our experiment efforts in this area are particularly promising.

In cooperation with staff of Ohara Hospital, Fukushima, Japan, we will study the pathology of tularemia nephritis in rodents vaccinated with organisms of low-virulence and challenged with virulent organisms. Preliminary experiments are in progress.

Serums, spinal fluids and brains from dogs observed for abortive rabies in Argentina are being tested for neutralizing antibodies against distemper virus. These studies, not complete, will be reported later. Serums and brains from wild skunks in a rabies epizootic area in North Dakota will be tested for neutralizing antibodies in attempts to demonstrate occurrence of and recovery from distemper in this highly susceptible species.

Publications:

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In press:

Owen, C. R.: Genus Francisella. Section in Bergey's Manual of Determinative Bacteriology (ed. 8). Baltimore, Williams & Wilkins.

Stoenner, H. G.: Differential diagnosis of leptospirosis in man. Rocky Mountain Med. J.

Serial No. NIAID-174
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Systematics and vector relationships of certain parasitic arthropods

Previous Serial Number: Same

Principal Investigator: J. M. Brennan

Other Investigators: C. M. Clifford, L. E. Hughes, E. K. Jones, J. E. Keirans, and C. E. Yunker

Cooperating Units:
Dr. T.H.G. Aitken, Rockefeller Foundation
American Museum of Natural History, New York
Mr. A. B. Amerson, Jr., Pacific Ocean Biological Survey Program, Smithsonian Institution
Bishop Museum, Honolulu, Hawaii
Dr. Alex Fain, Institute of Tropical Medicine, Antwerp, Belgium
Dr. C. O. Handley, Smithsonian Museum/Army Project
Dr. H. Hoogstraal, NAMRU-3, Cairo, Egypt
Dr. J. K. Jones, University of Kansas, Lawrence
Dr. R. B. Loomis, California State College, Long Beach
Dr. F. Lukoschus, Katholieke Universiteit, Nijmegen, The Netherlands
MARU, Canal Zone
Dr. D. Sonenshine, Old Dominion University, Norfolk, Va.
Dr. V. J. Tipton, Brigham Young University, Provo, Utah
Dr. R. Wenzel, Field Museum of Natural History, Chicago

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	5 8/12		3/12	5 11/12
Professional:	3 6/12		3/12	3 9/12
Other:	2 2/12			2 2/12

Serial No. NIAID-174

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
03-005-1 (PL-480)	C. M. Clifford H. G. Stoenner	NAMRU-3 Cairo, Egypt	H. Hoogstraal	$16,930

Project Description:

Objectives:

Taxonomy of medically important parasitic arthropods and their colonization.

Methods employed:

Standard and/or adapted field and laboratory techniques for collecting, handling and colonizing parasitic arthropods. Collaboration with appropriate sections at RML and outside agencies. Elucidation of systematic problems and identification, principally of acarines, as dictated by potential medical importance, as requested by health and bio-medically oriented organizations and investigators, and as stimulated by museums and other institutions sponsoring expeditions.

Major findings:

1. Taxonomy of ticks (Clifford, Keirans, and Jones). The tick collection of the Smithsonian Institution, National Museum of Natural History (including the F. C. Bishopp collection) is being transferred to RML where it will be housed and curated as is the National chigger collection which was moved here in 1966. Transfer of Dr. Hoogstraal's tick collection and library has been continued.

Numerous identifications of ticks from all parts of the world were provided, as requested, 20 or more agencies including USDA, SEATO, WHO, schools and research institutions.

Systematic studies leading to a revision of Ixodes subgenera, identification of immature stages of Ixodes in the Western Hemisphere, ticks of Venezuela, and ticks of Nepal are in progress.

2. Taxonomy of parasitic mites, exclusive of chiggers (Yunker). [illegible] of about 400 collections of dermanyssoid mites of Venezuela have been mounted and identified. The remainder, a nearly equal amount, is being prepared for study. Forty-five species, of which 21 (including 1 genus) are new, have already been recognized. Other large collections under study are dermanyssoid mites of Central America and Surinam, as well as macronyssid bat-parasites of Colombia and parasitic acarines of Bolivia.

Identification of mites collected by Dr. Hoogstraal and associates in Africa and Indonesia was continued. In addition, requested identifications, specimens, and biological or control information were provided about a dozen institutions and a number of private individuals.

Emphasis was placed in updating a lengthy chapter on mites for "Diseases of Laboratory Animals" (R. J. Flynn, Argonne National Laboratory) to be published in 1971.

3. Taxonomy of chiggers (Brennan). Slide mounts of the large Venezuelan collection were finally completed so that 18 to 20 thousand specimens are now catalogued and gradually undergoing identification. About one-fourth of the material is identified and already 20 to 25 undescribed species have been noted and many new host and distributional records have been established for other species.

Studies are continuing on Neotropical chiggers with particular reference to northern Brazil, Bolivia, Surinam and Colombia in order to provide more logical generic concepts and a better understanding of older, but poorly defined, species; a continued study of chiggers of the Central Pacific islands as part of the Smithsonian Pacific Ocean Biological Survey Project is nearly completed.

Numerous identifications were made for organizations including the American Museum of Natural History, Smithsonian Institution, U.S. Department of Agriculture, Rockefeller Foundation, Field Museum of Natural History, and schools and individuals.

4. The number of published new species and genera (in parentheses) according to zoogeographic regions are:

	Ethiopian	Palearctic	Oriental	Neotropical
Ticks	1	1	1	
Mites	3(2)			12(4)

Significance to bio-medical research and the program of the Institute:

Where arthropods are concerned in diseases in nature communicable to man, their systematic studies are basic to all other research emanating therefrom. Fortunately, the taxonomically oriented staff at RML is uniquely competent in the systematics of ticks and parasitic mites, lice, and, to a more limited extent, of biting flies. As authorities in the fields, we provide service within and outside our organization including identifications, consultation and information with respect to parasitic arthropods, and maintenance of certain species in colonies and their supply as requested.

Currently colonies of 11 species of ixodid and 5 of argasid ticks are maintained, also colonies of mites, Ornithonyssus bacoti, Haemolaelaps glasgowi group, and Eutrombicula splendens are available. Colonies of 2 important mosquitoes, Aedes aegypti and Culex tarsalis, are maintained.

Major and smaller sophisticated studies of appropriate acarine groups contributed to clarification of the status of species of biomedical or potential medical importance.

Proposed course of project:

Continuation of above investigations and services with expansion in any area as dictated by need.

Honors and Awards:

Dr. C. M. Clifford

Appointed as Adjunct Professor of Zoology, Washington State University.

Publications:

Brennan, J. M.: Colicus, a new Neotropical genus with descriptions of two new species and a key to included species (Acarina: Trombiculidae). J. Med. Entom. 7: 271-273, 1970.

Kohls, G. M., Clifford, C. M., and Hoogstraal, H.: Ixodes (Scaphixodes) mitchelli, n. sp. (Acarina: Ixodidae), a tick parasitizing pheasants and partridges in high mountains of Nepal. J. Med. Entom. 7: 348-352, 1970.

Yunker, C. E.: New genera and species of Ewingidae (Acari: Sarcoptiformes) from pagurids (Crustacea), with notes on Ewingia cenobitae Pearse, 1929. Rev. Zool. Bot. Afr. 81: 237-254, 1970.

Brennan, J. M.: Chiggers from the Bolivian-Brazilian border (Acarina: Trombiculidae). J. Parasit. 56: 807-812, 1970.

Brennan, J. M.: A review of Neoschoengastia colombiae Boshell and Kerr, 1942 with descriptions of two new species misidentified with it (Acarina: Trombiculidae: Colicus). J. Med. Entom. 7: 489-493, 1970.

Brennan, J. M. and Vercammen-Grandjean, P. H.: Revival of the monotypic genus Boshellia Ewing, 1950 (Acarina: Trombiculidae). Bull. Southern Calif. Acad. Sci. 69: 100-103, 1970.

Brennan, J. M.: Two new genera and a new species of Neotropical chiggers (Acarina: Trombiculidae). Ann. Entom. Soc. Amer. 63: 1694-1696, 1970.

Keirans, J. E., Clifford, C. M., and Hoogstraal, H.: Description of the male and immature stages of *Ixodes* (*I.*) *werneri* Kohls (Acarina: Ixodidae), a parasite of *Rattus* in mountains of Palawan, Malaya, and Java. J. Med. Entom. 7: 605-608, 1970.

Uilenberg, G., Clifford, C. M., and Hoogstraal, H.: *Ixodes albignaci* Uilenberg and Hoogstraal: Description of the male, nymph, and larva, and distribution and hosts in Madagascar (Acarina: Ixodidae). J. Med. Entom. 7: 611-614, 1970.

Philip, C. B. and Coscaron, S.: Comparative terminalia of certain Nearctic and Palaearctic Chrysopsine flies. Ann. Entom. Soc. Amer. 64: 157-162, 1971.

Brennan, J. M.: Three new chiggers from spiny rat, *Proechimys guyannensis*, in northern Brazil (Acarina: Trombiculidae). Ann. Entom. Soc. Amer. 64: 212-215, 1971.

Keirans, J. E. and Kohls, G. M.: *Ixodes philipi* n. sp. (Acarina: Ixodidae), a sea bird parasite in Japan. J. Med. Entom. 7: 725-726, 1970.

Philip, C. B.: Further notes on Oriental Tabanidae (Diptera). IV. Descriptions of *Cydistomyia* and *Tabanus*, and other records, particularly from India and Sikkim. In H. D. Srivastava Commemoration Volume. Lucknow, India, 1970, pp. 443-452.

In press:

Stout, I. J., Clifford, C. M., Keirans, J. E., and Portman, R. W.: *Dermacentor variabilis* (Say) (Acarina: Ixodidae) established in southeastern Washington and northern Idaho. J. Med. Entom.

Philip, C. B.: Some nomenclatorial changes in the Family Rickettsiaceae in anticipation of a new edition of Bergey's Manual of Determinative Bacteriology. Proc. 8th Int. Cong. Trop. Med. Malar.

Philip, C. B. and Coscaron, S.: New Neotropical Tabanidae. II. Three primitive undescribed pangoniine-like flies of unusual interest from Chile. Papeis Avulsos de Zoologia

Rupeš, V., Yunker, C. E., and Wilson, N.: *Zibethacarus*, n. gen. and three new species of *Dermacarus* (Acari: Labidophoridae). J. Med. Entom.

In press (cont'd)

Yunker, C. E.: Acarina other than ticks. In Flynn, R. J. (Ed.): Diseases of Laboratory Animals.

Radovsky, F. J. and Yunker, C. E.: *Xenarthronyssus furmani*, n. gen., n. sp., with two subspecies of Dasyponyssidae, parasites of armadillos (Acarina: Mesostigmata). J. Med. Entom.

Brennan, J. M. and Lukoschus, F.: Parasitic mites of Surinam. VIII. A new genus and species of chiggers, *Fauranius atecmarta*, and additional records of species (Acarina: Trombiculidae). Bull. Southern Calif. Acad. Sci.

Brennan, J. M.: Endoparasitic chiggers. VI. *Neoschoengastia esorhina* n. sp., a second intranasal chigger of birds (Acarina: Trombiculidae). J. Parasit.

Brennan, J. M. and Amerson, A. B.: Five new species and additional records of chiggers from the Central Pacific (Acarina: Trombiculidae). J. Parasit.

Keirans, J. E. and Jones, E. K.: Description of the immature stages of *Ixodes* (*I.*) *sinaloa* Kohls and Clifford (Acarina: Ixodidae), from rodents in Mexico and Nicaragua. Acarologia.

Clifford, C. M., Keirans, J. E., and Hoogstraal, H.: *Ixodes* (*Ixodes*) *nuttallianus* Schulze: Redescription of female, description of male, and hosts and ecology in Nepal (Acarina: Ixodidae). J. Med. Entom.

Clifford, C. M., Hoogstraal, H., and Kohls, G. M.: *Ixodes hyatti*, n. sp., and *I. shahi*, n. sp. (Acarina: Ixodidae), parasites of pikas (Lagomorpha: Ochotonidae) in the Himalayas of Nepal and West Pakistan. J. Med. Entom.

Serial No. NIAID-175
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Allergy, its mechanisms and role in disease

Previous Serial Number: Same

Principal Investigators: J. J. Munoz, R. K. Bergman, J. E. Coe, and D. L. Lodmell

Other Investigators: W. J. Hadlow, S. C. Harris, G. C. Lavelle, and R. E. Race

Cooperating Units: Dr. R. Hunter, Dept. of Pathology, Univ. of Chicago Medical School, Chicago, Ill.
Dr. Ulf Nilsson, Univ. of Pennsylvania Medical School, Philadelphia
Scripps Clinic and Research Foundation, La Jolla, Calif.: Dr. C. Clark and Dr. Hans Spiegelberg
Dr. K. Takemoto, LVD, NIAID, NIH, Bethesda, Md.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	10 7/12		9/12	11 4/12
Professional:	3 7/12		9/12	4 4/12
Other:	7			7

Project Description:

Objectives:

The aims of this project have been gradually changed to involve our section more extensively in the study of slow viral diseases. The general broad aims are: 1) to study the fundamental basis of hypersensitivity and its role in disease, 2) to learn more about the function of immunoglobulin classes in various animal species, and 3) to investigate antibody response and body defense mechanisms in slow virus infections.

Methods employed:

Methods employed are typical immunological, biochemical, biophysical and serological procedures.

Major findings:

1. *Hypersensitivity reactions to insulin (Munoz)*. Delayed hypersensitivity to bovine insulin, and to the A and B polypeptide chains derived from it was induced in Hartley guinea pigs. The determinants involved in sensitization were shown to be different in the intact molecule than in the peptide chains (see last year's report). This was shown by skin test reactions in sensitized and in sensitized and subsequently desensitized guinea pigs. This year we studied by the macrophage migration inhibition test (MIF), the reactions obtained with macrophages derived from guinea pigs sensitized with insulin-DNP-insulin, and B polypeptide chain. The results can be summarized as follows: a) Macrophages from insulin or DNP-insulin sensitized guinea pigs are inhibited from migrating by insulin, B chain and A chain. b) Macrophages from guinea pigs desensitized with insulin migrated in the presence of insulin and B chain but not as well as the control without the antigen (probably only partially desensitized). c) Macrophages from sensitized guinea pigs desensitized with B chain were not fully desensitized, perhaps due to insufficient amounts of B chain used in desensitization. d) *In vitro* desensitization experiments in which cells from sensitized pigs were exposed to different antigens and then washed and tested by the MIF test showed consistently that sensitive cells once exposed to the sensitizing or cross reacting antigen did not recover their ability to migrate.

It was interesting to note that no change in the glucose metabolism (blood sugar levels or glucose tolerance) nor histopathologic change in the pancreas was observed in guinea pigs highly sensitive to bovine insulin. It was noteworthy that the conjugation of insulin to DNP did not change its specificity very markedly, probably because only one DNP molecule can be attached to the insulin molecule.

2. *Antibody response in mink inoculated with Aleutian disease (AD) virus (Lodmell, Bergman, Hadlow and Race)*. The response of sapphire (highly susceptible to AD) and pastel (rather resistant to AD) mink to antigenic stimulation with goat erythrocytes (G-RBC) was studied at various stages after AD virus inoculation and the following results were obtained.

a. *Development of antibody plaque forming cells in lymphoid organs of mink receiving virus one day after primary immunization*. There were essentially no differences between the number of 19S and 7S plaque forming cells (PFC) in spleen and lymph nodes of pastel and sapphire mink sacrificed at various times after primary immunization. After a booster dose of antigen, peak 19S and 7S PFC responses were generally attained earlier in sapphire mink. In the anamnestic response in pastels greater numbers of 7S PFC were observed. The hemolysin response was slightly higher in pastel mink; the higher titer was due mainly to 2-mercaptoethanol (2 ME)-sensitive (19S) antibody, since the titers of 2 ME-resistant (7S) antibody were comparable in both color phases. In addition, abdominal lymph nodes (ALN) of both color phases contained a greater number of 19S and 7S PFC than spleens, and the latter had more than peripheral lymph nodes (PLN). Of 2 mink of each color phase that became AD affected during the experiment, the pastels had greater quantities of PFC and higher hemolysin titers.

b. PFC responses and hemolysin titers of mink inoculated with G-RBC 47 days after AD virus inoculation. At time of inoculation with G-RBC all sapphires, but only 9 of 20 (45%) pastels, were affected with AD. Neither primary nor anamnestic 19S or 7S PFC was detected in spleens of AD-affected sapphires. Both unaffected and AD-affected pastels had considerable numbers of 19S and 7S splenic PFC, but more were detected in unaffected mink. In contrast to the complete lack of splenic PFC in AD-affected sapphires, primary and anamnestic 19S and 7S PFC were detected in some ALN. All pastel mink, regardless of their clinical condition, had primary and anamnestic 19S and 7S PFC in ALN. Whereas the greatest number of PFC were enumerated in ALN of unaffected pastels, AD-affected pastels were more responsive than sapphires affected with AD. Few primary or anamnestic 19S and 7S PFC were detected in PLN of either AD-affected sapphire, pastel mink or unaffected pastels. Primary total and 2-ME resistant serum hemolysin titers of AD-affected pastels and sapphires were very similar. Both hemolysin titers were greater in unaffected pastels. Few AD-affected sapphires possessed total or 2-ME resistant hemolysins after booster inoculation. Both hemolysin responses were markedly higher in the pastels, but there was little difference in titers between unaffected or AD-affected pastel mink.

3. Jugular catheterization of mink (Bergman, Lodmell and Hadlow). A method was devised to insert a polyethylene catheter into the jugular vein of mink to facilitate intravenous injections or withdrawal of blood samples at timed intervals. The method has been successfully applied to over 30 mink. It has been used during short term experiments while an animal has remained under anesthesia for 2 to 3 hours and it has also been successfully used on mink during a 24-hour period to bleed unanesthetized animals. By inserting a long needle through the catheter, injections can be made directly into the blood stream without contaminating the walls of the catheter. Numerous small blood samples can be taken at timed intervals or one large sample (50 to 55 ml) in a single withdrawal.

4. Measurement of adrenal steroids and catecholamines in blood of experimental animals (Bergman). To examine possible interrelationships between the functions of the neuroendocrine system and immune mechanisms in animals, we have tried to develop and adapt assay procedures for determining adrenal steroids and catecholamines in the blood. Techniques, utilizing centrifugal chromatography, are being developed to separate hydrocortisone and corticosterone in methylene chloride extracts of plasma. The separated steroids can then be eluted from the silica gel column and quantitated by fluorescence. This method promises to be much faster and more direct than any of the currently available methods, and requires only about 3 ml of plasma.

The method which shows the greatest promise for assaying catecholamines utilizes a two-step chromatography to separate epinephrine and norepinephrine from plasma. By using radiolabeled catecholamines we have been able to follow the fractionation in chromatography and to test methods to improve the recovery of the amines. The two catecholamines are quantitated separately by a differential fluorometric assay based on the trihydroxyindole reaction. With dependable assay methods for these hormones it may be possible to relate various physiological, pathological and immunological changes in animals with alterations in the neuroendocrine system, that may occur in such diseases as AD of mink and in various hypersensitivity phenomena.

5. Evaluation of reticuloendothelial system (RES) phagocytic activity of normal and AD-affected mink (Lodmell, Bergman, Hadlow and Race). This work was done to compare clearance of ^{125}I-microaggregated human serum albumin (^{125}I-HSA) and colloidal carbon from the bloodstream of normal and AD-affected mink and to study the phagocytic role of the major organs of the RES. Normal mink and mink exhibiting early clinical signs of AD removed comparable amounts of ^{125}I-HSA from the bloodstream. There was, however, a marked difference in the amount of ^{125}I-HSA/mg tissue of the two groups. Significantly greater amounts of ^{125}I-HSA were found in spleen, liver, and bone marrow of normal mink than in those with early AD. Radioactivity/mg of tissue for other organs tested was quite similar. In contrast to similar clearance rates of ^{125}I-HSA, colloidal carbon was removed somewhat more quickly from the bloodstream of normal mink.

AD-affected mink in advanced disease had a reduced capacity to clear both ^{125}I-HSA and carbon from the bloodstream. Approximately the same radioactivity/mg tissue was detected for all tissues of early and chronically AD-affected mink. The data appear to indicate that there is, indeed, reduced capacity for phagocytic cells of AD-affected mink to phagocytize foreign particles introduced into the bloodstream.

6. Mink interferon: In vivo and in vitro studies (Lodmell). The present study was initiated to determine if interferon could be produced in mink inoculated with AD virus and to measure the capacity of mink cells *in vitro* and *in vivo* to respond to known inducers of interferon. Six primary mink cell cultures and one established mink cell line have been used in attempts to demonstrate cellular interference and interferon production with various known interferon inducers, such as the annealed copolymer of polyinosinic-polycytidylic acid (rI·rC), statolon, and endotoxin. Under conditions tried (different cell cultures, various concentrations of inducers with or without DEAE-dextran or neomycin, various lengths of incubation, preheated rI·rC, different diluents, and growth of cell monolayers in the presence of rI·rC) we have failed to demonstrate either cellular resistance or interferon production, as assayed by plaque reduction with vesicular stomatitis virus (VSV). Nonetheless, a more sensitive assay system and a number of other experiments are in progress or planned which may produce more affirmative results.

The *in vivo* studies have consisted of intravenous injections of various concentrations of rI·rC and statolon. Several of these experiments are still in progress, but preliminary results have shown that both inducers initiated low levels of interferon production. Of the two, the rI·rC appears to be much more efficient. Assays for interferon in AD-inoculated mink are in progress.

7. Studies on mink immunoglobulins (Coe). In addition to the IgG and IgM which we previously described (Annual Report, 1969), mink IgA has been characterized. This protein has L chain determinants common to the other Ig and also unique heavy chain determinants. IgA was found in serum, saliva, enteric contents, and urine, chiefly in the last two; that in enteric contents and urine was antigenically identical to serum IgA. Also, IgA in various fluids had similar electrophoretic mobility and sedimentation value (≃12.5S); however, in contrast to the IgA in other fluids, the S value of serum IgA changed mainly to 7S after mild reduction and alkylation. This finding indicates possible differences in the bonds forming the IgA polymers in various fluids. IgA antibody to keyhole limpet hemocyanin (KLH) was detected in serums obtained from KLH immunized mink and preliminary observations suggest marked increases of IgA in serums of AD-infected mink.

Extensive analysis of normal and immune mink serum for another 7S Ig class has not been successful. However, a new IgG subclass of fast γ-slow β mobility, which has been identified, has unique H chain determinants, but also shares H chain determinants with the major IgG class. Limited observations indicate that this subclass IgG is markedly increased in serums from mink with AD.

Mink were found to have two antigenically distinct L chains of which one (light chain a) had unique determinants not present on the other light chain group (b); light chain b did not appear to contain any unique determinants. Preliminary evaluation indicates that these two light chain types are present in all mink (male and female, sapphire and pastel) and therefore are not allotypes. These two L chain populations are probably not analogous to κ and λ human L chain types, because of shared antigens. However, the two light chain populations may be comparable to the human L chain subtypes such as oz (+ and -). Specific antiserums for these mink L chains have been prepared and will provide further information about the monoclonal-like Ig abnormalities present in serums from some AD mink.

8. Studies on production of antipolyoma virus antibody in hamsters (Coe). (Studies in collaboration with Dr. K. Takemoto, LVD, NIAID, Bethesda). Neonatal and adult hamsters were inoculated with polyoma virus or polyoma tumor cells and the appearance of serum antibody to T or V antigens was assayed by fluorescent microscopy. Fluorescein-labelled antiserums specific for hamster $7S\gamma_1$, $7S\gamma_2$, γA and γM-Ig were used so that antibody activity could be related to a known Ig class. Preliminary observations suggest differences in the adult and neonatal Ig response to polyoma virus, a virus which causes tumors in neonatal hamsters but not in normal adult hamsters. For instance, neonatal hamsters had $7S\gamma_2$ antibody (7 days post-injection) to the V antigen and subsequently (3 weeks post-injection) $7S\gamma_1$ and $7S\gamma_2$ antibody to the T antigen. Adult hamsters produced $7S\gamma_2$ and IgM antibody to the V antigen and only $7S\gamma_2$ antibody to the T antigen. However, adults inoculated with polyoma tumor cells produced antibody to T antigen in the $7S\gamma_1$, $7S\gamma_2$ and IgM Ig. The interaction of $7S\gamma_1$ antibody (noncomplement fixing) and $7S\gamma_2$ antibody (complement fixing) may have contrasting effects (i.e., blocking or cytotoxic, respectively) in the immune response to oncogenic tumors.

By the use of fluorescein-labelled antiserums to sheep Ig, an indirect fluorescence assay for sheep antibody to progressive pneumonia virus has been developed, and antigens common to this "slow" virus and Visna virus have been demonstrated.

9. Studies on guinea pig IgA (Coe). A secretory Ig (IgA) of the guinea pig has been characterized further. This Ig was found in greater concentration (10 to 20X) in guinea pig colostrum than in serum. IgA of various fluids sedimented mainly around 12S although serum and enteric IgA were heterogeneous in size and extended into the 7S region. Colostral IgA appeared to have a minor 16S component in addition to the major 12S population. The IgA from various fluids appeared antigenically similar when tested with rabbit serums specific for the IgA of enteric contents or saliva, although serum IgA was electrophoretically faster than colostral IgA. Antibody activity was demonstrated in serum IgA after injection of

guinea pigs with HEA or BSA in Freund's adjuvants, provided the guinea pigs had previously received 1% antigen in the drinking water.

10. Immune response in the painted turtle (Coe). Utilizing our existing autoradiographic techniques for antibody detection, we studied antibody production in the turtle to purified protein antigens. Four different serum immunoelectrophoretic-precipitin lines developed specific antigen binding capacity after immunization. These four proteins differed in their electrophoretic mobility (γ to β), their size (19S to 7S) and the time at which antibody synthesis began after immunization. The slowest gamma globulin in turtle serum was found to be an iron-binding protein rather than an immunoglobulin.

11. Studies with antibody produced in bullfrogs (Coe). The results of previous studies at the RML indicated that the specificity of bullfrog antibody was different than conventional mammalian antibody, i.e., bullfrog antiserums detected antigenic determinants on mammalian proteins which were not recognized by mammalian antiserums to the same proteins. (Presumably the greater phylogenetic distance of the bullfrog permits its recognition of determinants common to mammals.) To detect new immunoglobulin subclasses and to develop antiserums with broad mammalian specificity (for homology studies), we immunized bullfrogs with various purified Ig from human and rodent serums. Preliminary data indicate that bullfrog anti-human IgA may be detecting a heretofore undefined IgA type in humans. In addition, bullfrogs have been immunized with purified human complement components (C_3, C_5) and these antiserums are being used for antigenic analysis of these complex proteins.

Significance to bio-medical research and the program of the Institute:

Studies on delayed hypersensitivity to insulin and its component peptide chains were conducted in an effort to uncover further similarities or differences between immediate and delayed hypersensitivity. The macrophage migration inhibition test confirmed that delayed hypersensitivity to bovine insulin and its component peptide chains can easily be induced in the guinea pig and furnish a simple model to study various fundamental aspects of development of cellular hypersensitivity.

Studies of antibody-forming cells in mink at various stages of AD virus have contributed to the understanding of how AD affects antibody forming cells and also the understanding of differences in susceptibility in pastel and sapphire mink. It is clear from the results obtained that once the virus infection is well-established no significant antibody response to new antigens could be demonstrated. This must be of great importance to the outcome of the disease. The mere presence of virus in [illegible] animal at the time of antigenic stimulation does not inhibit antibody [illegible]. The inhibition occurs after clear symptoms of AD have developed. The depressed phagocytic activity in these sick animals may be a reflection of a generalized deterioration of the immune processes.

Preliminary studies on interferon and on determination of steroids are aimed at understanding better the pathogenesis of AD in mink.

Since little has been done with the study of mink immunoglobulins the studies along these lines have enriched our knowledge in this field. In addition to showing the great similarity of immunoglobulins to other mammals, some peculiarities in the mink systems are becoming evident.

The studies in hamsters and polyoma tumor cells are aimed at explaining the reasons for the great susceptibility of neonatal hamster to the development of tumor. An understanding of the role played by the various types of immunoglobulins such as complement fixing ($7S\gamma_1$) vs. non-fixing ones ($7S\gamma_2$) would be of great help in understanding the role played by antibodies in development of a tumor.

Studies on the immunoglobulins of various other animals have enlarged our knowledge of these interesting proteins.

Proposed course of project:

As stated in last year's report, the loss of personnel has had an effect on the direction and scope of the research in this section. This condition will persist through the next fiscal year. We will continue to emphasize the studies with slow virus infections in which now three of our four professionals are deeply involved. Studies on immunoglobulins in various species will be continued.

Honors and Awards:

Dr. J. J. Munoz

Continued as Associate Editor of the Journal of Immunology.

Courtesy appointment as Faculty Affiliate, University of Montana.

Serve as member of the Council for the Mid-Winter Immunologist Conference.

Dr. J. E. Coe

Gave seminar, December 1970, at the University of Montana entitled "Immune response in lower vertebrates".

Courtesy appointment as Faculty Affiliate, University of Montana.

Publications:

Coe, J. E.: Specificity of antibody produced in the bullfrog (*Rana catesbeiana*). *J. Immun.* 104: 1166-1174, 1970.

Clark, C. and Munoz, J.: Delayed hypersensitivity to insulin and its component polypeptide chains. *J. Immun.* 105: 574-583, 1970.

Coe, J., Peel, L., and Smith, R. F.: Biologic activities of Peromyscus $7S\gamma_1$ and $7S\gamma_2$ globulins. *J. Immun.* 105: 1006-1010, 1970.

Coe, J. E. and Peel, L. F.: Antibody production in the bullfrog (*Rana catesbeiana*). *Immunology* 19: 539-550, 1970.

Coe, J. E.: Antigenic variation of $7S\gamma_1$, $7S\gamma_2$, and γM globulins within the genus *Peromyscus*. *J. Immun.* 106: 34-40, 1971.

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Serial No. NIAID-177
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immune prophylaxis of mycobacterial infections

Previous Serial Number: Same

Principal Investigators: R. L. Anacker and E. Ribi

Other Investigators: W. D. Bickel, W. R. Brown, G. Goode, S. C. Harris, R. H. List, and R. F. Smith

Cooperating Units: Dr. W. R. Barclay, AMA, Chicago
Dr. A. Bekierkunst, Hadassah Medical School, Jerusalem, Israel
Dr. W. Brehmer, Robert Koch Institute, Berlin, Germany
Dr. M. Goihman-Yahr, Hospital Varga, Caracas, Venezuela
Dr. M. Kato, Res. Institute of Tuberculosis, Osaka, Japan
Dr. D. Koch-Weser, Harvard School of Pub. Health, Boston
Dr. J. Matsumoto, Research Institute of Tuberculosis, Toyonaka, Osaka, Japan
Dr. H. Wolochow, Naval Biological Laboratory, Oakland
Dr. K. Yamamoto, Hokkaido University, Sapporo, Japan

Man Years:	NIAID Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	10 9/12		11/12	11 8/12
Professional:	4 7/12		11/12	5 6/12
Other:	6 2/12			6 2/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
N00-14-69-A-1001	E. Ribi	Naval Biol Lab, Oakland, Calif.	H. Wolochow	$50,600

Serial No. NIAID-177

Project Description:

Objectives:

To develop an effective nonliving antituberculosis vaccine from purified fractions of mycobacteria, to study the nature of the host responses to these fractions, and to determine the relationship of delayed hypersensitivity to immunity.

Methods employed:

A variety of physical, chemical, and immunologic techniques was used to prepare and characterize cell walls and other fractions of several mycobacterial species and strains and to assay the response of experimental animals to these mycobacterial fractions. Protective potency of these preparations was determined in mice, monkeys, and guinea pigs challenged by aerosol with Mycobacterium tuberculosis H37Rv.

Major findings:

1. Vaccine trials in monkeys. Evaluation of the BCG cell-wall vaccine in rhesus monkeys has been continued in collaboration with the staff at the Naval Biological Laboratory. The protection afforded rhesus monkeys vaccinated in an earlier experiment with the cell-wall vaccine by different routes was compared with that provided by living BCG by the same routes, i.e., intravenous, intramuscular, and subcutaneous.

Living BCG, like cell walls, induced pathological changes in the recipients. Lesions at the site of vaccination were not grossly observed in monkeys vaccinated intramuscularly with 1.0 mg BCG or subcutaneously with 0.1 mg BCG, but nodules 2 to 3 cm in diameter developed in animals vaccinated with 1.0 mg BCG subcutaneously. These nodules later ulcerated and drained. Animals vaccinated intravenously were affected systemically; minor but definite inflammatory changes were observed in histologic sections of the lungs, and the size and number of germinal centers in the spleens were increased. Animals vaccinated intravenously also lost weight during the immediate postvaccination period.

Monkeys in the various groups differed greatly in their development of delayed hypersensitivity to PPD after vaccination. At the time of challenge none of the 6 animals given 0.1 mg of BCG subcutaneously reacted to PPD, and only 2 of the 6 given 1.0 mg subcutaneously gave a positive response. In contrast, 5 of 6 animals in each of the groups vaccinated with 1.0 mg of BCG either IM or IV reacted to challenge with PPD, and 8 of the reactions were very strong.

These monkeys were autopsied 10 weeks after airborne exposure to virulent tubercle bacilli. A numerical score was assigned to each animal according to the extent of grossly observable tuberculous disease in the lungs, tracheobronchial nodes, and abdominal organs; a score of 0 was assigned to an animal with no obvious disease and a score of 100 to an animal with maximal disease. The mean scores were 62.5 for control animals, 0 for monkeys vaccinated intravenously, 35.0 for animals vaccinated either with 0.1 or 1.0 mg subcutaneously, and 38.3 for animals vaccinated intramuscularly. These scores are roughly comparable to those obtained for monkeys vaccinated with cell walls by these routes.

2. Tuberculoimmunity and delayed hypersensitivity. Since some workers in the field of the immunology of tuberculosis believe that delayed hypersensitivity is correlated with immunity, several experiments were performed to determine whether the simple footpad test for delayed hypersensitivity of immune mice could replace the time-consuming and relatively expensive protection test presently employed at RML. As suggested by others, mice were given nonviable vaccines of varying potency, a booster dose of viable BCG several weeks later to accelerate the development of delayed hypersensitivity, and then the footpad test several days later. For comparison, mice given the same vaccines were challenged by the airborne route and protection was evaluated in the standard way. For the most part, delayed hypersensitivity produced by this regimen was minimal; no correlation between hypersensitivity and resistance could be demonstrated.

It was reported previously that mice given "immune" spleen cells and a small booster dose of BCG cell walls were always better protected against infection than were mice given "normal" spleen cells and the same quantity of BCG cell walls. However, the difference between groups was not always statistically significant, due in part to the response of recipients of normal spleen cells to BCG cell walls. To reduce this response we used mice irradiated with 750-800 R and supplemented with bone marrow cells as a substitute for normal recipients. In 2 experiments, differences between groups of irradiated mice were about the same as noted with nonirradiated mice. Apparently, among the population of normal spleen and bone marrow cells, there are cells, which after stimulation with the "booster" dose of cell walls, can afford significant resistance to the recipient. Obviously, our experimental model will have to be modified further to demonstrate unequivocally the role of lymphocytes in immunity.

Dr. Goihman-Yahr, using BCG cell walls and protoplasm prepared at RML, studied the cross reactivity between BCG and M. leprae in guinea pigs sensitized with lepromin or BCG. BCG cell walls provoked delayed skin reactions in lepromin-sensitized animals, but the protoplasmic fraction elicited no reaction whatsoever although protoplasm evoked reactions in

BCG sensitized guinea pigs. This result was contrary to one obtained earlier with a BCG protoplasmic fraction prepared in another laboratory; cross reaction in this case was attributed to contamination of the fraction with cell walls. Dr. Goihman-Yahr's latest results demonstrate that BCG cell walls, but not protoplasm, contain antigen(s) related to those found in M. leprae.

Dr. Koch-Weser, with our collaboration, investigated the relationship between acid fastness and immunogenicity in M. tuberculosis H37Ra. Cell walls from both normal acidfast bacteria and those rendered nonacidfast by growth in a medium containing ethambutal protected mice equally well against airborne infection. Apparently the properties of acidfastness and immunogenicity are unrelated.

The question whether pulmonary granulomatous changes which develop after intravenous vaccination with oil-treated cell walls are essential to acquired resistance has not been answered. As a preliminary experiment, vaccine was inoculated into a portion of the left lung of rhesus monkeys by cardiac catheterization techniques under fluoroscopic control to determine whether granulomas would develop only in the inoculated lung. Of the 2 animals successfully vaccinated in this manner, 1 had the cellular reaction confined primarily to 1 lobe and the other to 2 lobes. Histologically, these lobes were densely infiltrated with macrophages and lymphocytes. The remaining lung tissue had only a patchy infiltrate which probably was induced by vaccine not removed during its first passage through the inoculated lobe. These results suggest that it may be possible, after certain modifications of the experimental procedures, such as reduction of the dose, to vaccinate monkeys so that cellular changes are limited to specific lobes. Monkeys could then be exposed by aerosol challenge and resistance compared in the inoculated and uninoculated portions of the lung.

To elucidate further the immune mechanism in tuberculosis of mice, we studied the ability of various immunosuppressive drugs to alter the delayed hypersensitivity response in the footpad and the protective response after intravenous inoculation of oil-treated cell walls was investigated. Varying doses of Cytoxan (CY; 2, 6, or 12 mg/kg/day) or Imuran (IM; 1, 5, or 10 mg/kg/day) were given in the feed from time of vaccination to challenge. At 4 weeks postimmunization, the footpad response to PPD was reduced only in those mice treated with 6 or 12 mg CY/kg/day. At 4 weeks postchallenge , protection among vaccinated drug-treated and control mice did not differ significantly.

4. Immunogenic and adjuvant activities of cell walls. A study to determine the relationship between protective and adjuvant properties of the tubercle bacillus is in progress. Previously we demonstrated that immunogenic cell walls could be separated into 2 fractions, neither of

which was immunogenic when given separately. However, immunogenicity was restored when the 2 fractions were recombined. Similar experiments to determine whether adjuvant activity parallels immunogenic activity are now underway. Results from a preliminary experiment indicated that adjuvant activity was lost when cell walls were fractionated as above and was not restored upon recombination of the 2 fractions. These results suggest that immunogenic and adjuvant activities of the tubercle bacillus reside in separate entities of the cell wall; however, additional experimentation is necessary to confirm this tentative conclusion.

4. Fractionation of mycobacteria. Studies to identify the active principles in Wax D and other lipid extracts with the aid of centrifugal microparticulate-bed chromatography were continued. Each of the above preparations contained 7 or more components. Our fractions which are most active in protection tests are still heterogeneous, and from current available information it is impossible to identify which entity or combination thereof is responsible for various host reactions elicited by the tubercle bacillus.

Although some single bands from chromatograms of Wax D could be isolated with equipment presently available, only submilligram quantities could be processed at one time. Reclaiming each of the components, after rechromatographing them 2 or 3 times, in quantities sufficient for testing remains a formidable task. However, comparison of the chromatograms with results of protection tests has helped us to devise more refined extraction procedures which eliminate some irrelevant components.

To circumvent the mechanical complexities involved in preparative centrifugal chromatography, we have recently explored 3 procedures for fractionating samples up to 50 mg in size. First, some success has been achieved by passing extracts through beds of microparticulate gel 2.7 cm in diameter and 2 cm long in brass columns under the influence of pressure rather than centrifugal force. Also large samples dissolved in chloroform-methanol have been passed through large columns of minute glass beads with highly porous surfaces or LH-20 Sephadex. Fractions containing as few as 2 components were collected in quantities suitable for bioassays. Analytical centrifugal chromatography was still needed to determine the degree of homogeneity of the eluted fractions.

Fractionation of the cord factor also was continued. Last year's report included results of chromatographic analyses of samples prepared by Dr. Kato of Osaka. These samples were slightly immunogenic in mice and also produced granulomatous changes. Since then, we have examined a sample prepared by Dr. Lederer and believed by him to be homogeneous and found by others to enhance resistance in mice and to provoke granulomata. Hopefully, analysis of this preparation would enable us to determine whether our lipid extracts were contaminated with cord factor and also to identify the component(s) which induced granuloma formation and acquired resistance. Unfortunately, numerous components were revealed in Dr. Lederer's preparation by centrifugal chromatography; some of these components appeared to be identical to some found in Wax D and other extracts.

5. Tuberculosis in marmosets. As work has progressed, it has become apparent that a less expensive and more convenient sub-human primate model than the rhesus model would be desirable. In a preliminary study, [illegible] marmosets (a 300-400 g South American primate) were exposed to an estimated dose of 25-40 viable units of M. tuberculosis H37Rv by aerosol. All animals died within 5-6 weeks with extensive tuberculous disease. Although gross lesions were found only in the thoracic cavity, acidfast bacilli were found in liver, spleen, and kidney. Because of the marmoset's susceptibility to tuberculosis, relative economy for experimentation and freedom from lung mites (which cause tubercle-like lesions in rhesus monkeys), it has a definite potential for becoming the sub-human primate model of choice.

6. Aerosol immunization of mice. In an attempt to deliver the cell wall vaccine directly to the target organ, the lung, a series of experiments involving aerosol immunization was begun. Mice were exposed to aerosols of BCG cell walls suspended in Tween-saline. Single exposures for times estimated to permit inhalation of 10-20 µg doses conferred no protection. Two such exposures 3 weeks apart also failed to stimulate resistance.

Significance to bio-medical research and the program of the Institute:

Previous experiments with mice demonstrated that the intravenous route for administrating both viable BCG and nonviable antituberculosis vaccines was far superior to other routes, and present results have shown the intravenous route to be dramatically better in the rhesus monkey as well. Although one cannot assume that humans would respond in the same manner, the data suggest that serious attention should be given to the possibility that vaccination procedures more effective than those currently used for man can be developed.

We have encountered many difficulties in our attempts to fractionate the complex lipids of the tubercle bacillus, but the means to obtain for the first time chemically pure components now appear to be available. Although much hard work still lies ahead, the prospects are promising that we will be able to obtain pure components, determine their biological activity, and in collaboration with others identify them chemically and structurally. Such a study will enable us to determine whether an effective immunogen can be separated from those components of the tubercle bacillus which promote the undesirable host responses of granuloma formation and tuberculin sensitivity. In unraveling the nature and role of these complex lipids we will develop a better understanding of disease processes and host responses in tuberculosis specifically, and perhaps chronic diseases in general.

Proposed course of the project:

Several lines of investigation will be continued in the coming year. First studies of the immunogenicity and other effects of the cell wall vaccine in several species in addition to the mouse will be pursued. An experiment is now in progress at the Naval Biological Laboratory to determine the duration of immunity to airborne infection of rhesus monkeys vaccinated with cell walls or live BCG. Soon, the immune response of marmosets given cell wall and live BCG vaccines will be investigated. Finally, the immunizing and sensitizing properties of cell walls and live BCG in guinea pigs will be compared.

In addition, a major effort will be made to purify various mycobacterial lipids and to determine their host-reactive properties. Also, further attempts will be made to immunize mice with nonviable vaccines by the airborne route.

Publications:

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Ribi, E., Brehmer, W., Milner, K. C., and Wicht, W. C.: Specific and nonspecific stimulation of resistance in mice against infection with *Mycobacterium tuberculosis* H37Rv. *Pneumonology* 142: 264-273, 1970.

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In press:

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Serial No. NIAID-178
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure and biologic activity of endotoxins

Previous Serial Number: Same

Principal Investigators: K. C. Milner and E. Ribi

Other Investigators: W. D. Bickel, D.Y.L. Leong (NIH Postdoctoral Fellow), and J. A. Rudbach

Cooperating Units:
Dr. J. Gander, University of Minnesota, St. Paul
Dr. E. Hausmann, State University of New York, Buffalo
Mrs. Kathleen E. Jackson, University of Montana, Missoula
Syracuse University, Syracuse, N.Y.: Dr. J. R. Vestal, and Dr. D. G. Lundgren
Dr. K. F. Meyer, University of California, San Francisco
Dr. M. Niwa, Osaka City University Med. School, Japan
Dr. A. M. Pappenheimer, Jr., Harvard Univ., Cambridge
Dr. M. R. Venneman, University of Texas, Austin
Dr. P. A. Ward, Armed Forces Institute of Pathology, Washington, D.C.
Dr. R. W. Wheat, Duke University, Durham, North Carolina
Dr. S. M. Wolff, LCI, NIAID, NIH, Bethesda, Maryland

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	7 8/12		6/12	8 2/12
Professional:	1 10/12		6/12	2 4/12
Other:	5 10/12			5 10/12

Project Description:

Objectives:

To investigate the features of composition and structure of bacterial endotoxins (ET) required for expression of characteristic host reactivity.

Methods employed:

A combination of biophysical, chemical, and immunological approaches has been used.

Major findings:

1. The studies of induced resistance to bacterial endotoxins (tolerance) referred to in earlier reports (1968, 1969) will be terminated during the next year unless fresh approaches promise new insights. In the meantime, we and others have shown that rapidly-developing *early* tolerance is a cellular, not a humoral, phenomenon. A form of *late* tolerance, which first appears several days after primary exposure, and is much more specific than early tolerance, appears to be mediated by antibody and can be transferred passively with serum, plasma, or primed spleen cells. Our studies were designed to determine whether the effective transfer factor is antibody to a special toxic conformation (antiendotoxin), or ordinary O antibody.

Through a variety of experiments we have shown that tolerance in rabbits and mice can be mediated by O antibody alone. Immunization with endotoxins from O antigen-deficient mutants has not produced appreciable *late* tolerance and has stimulated very little production of transfer factor, either to the homologous or to wild-type endotoxins. The latter have been highly effective in both respects. Late tolerance and passive transfer have been specific, even among products from such related organisms as colon bacilli and salmonellae. *Early* tolerance, in contrast, appears to show no interendotoxin specificity; and we have been unable to achieve passive transfer with nonpyrogenic sera drawn within 24 hours of a single exposure to endotoxin.

In collaboration with Dr. Pappenheimer, we have explored his suggestion that *late* tolerance might be an anaphylactic type of response, which depresses the temperatures of guinea pigs. This mechanism has not been completely ruled out, but we have not found it to play any part in the modified fever responses of actively or passively tolerant rabbits.

2. A group from Syracuse University sought our collaboration in studies of the cell envelope of *Thiobacillus ferrooxidans*, a gram-negative facultative autotrophe of economic and public health importance capable of utilizing reduced iron, sulphur, or glucose as energy source. They had preliminary indications that lipopolysaccharides (LPS) from this bacterium might have endotoxic properties. We confirmed this, but activities were not of the same order as those of LPS from Enterobacteriaceae. Chemical and antigenic composition, however, and endotoxic potency varied markedly with the substrate supplied for growth.

Extracts from cells grown on iron (Fe-LPS), sulphur (S-LPS), and glucose (Glu-LPS) contained little or no phosphorus, no dideoxysugars (except KDO), 9 to 14% fatty acids, and relatively large amounts of methyl pentose. Fe-LPS was by far the most potent endotoxin, yet its chemical composition was essentially that of Glu-LPS. S-LPS and Glu-LPS had about the same endotoxic potency, but differed sharply in content of hexosamine, KDO, and heptose. Quantitative precipitin tests and Ouchterlony gel diffusion studies showed that S-LPS was less immunogenic than the other two, and that each contained one or more unique, as well as some shared, antigenic determinants.

3. In collaboration with Mrs. Jackson, we studied the distribution and persistence of lipopolysaccharides in mice. Radioisotope tracing was used to detect endotoxins or their products, and two methods of labeling were compared. Lipopolysaccharide extracted from the cell walls of Escherichia coli 0113 was labeled externally with ^{51}Cr by a standard method. An internally labeled preparation was extracted from the cell walls of E. coli grown in a medium containing ^{14}C in the carbon source. Large numbers of mice were inoculated intravenously with graded sublethal doses of each preparation and sampled at weekly intervals for 12 weeks. Organs, tissues and extracts thereof were assayed for radioactivity, with counts being converted to quantitative estimates of endotoxin.

The principal findings were: a) The two methods of labeling gave qualitatively and quantitatively similar values. b) Adjustment of doses between submicrogram and overtly toxic amounts led to no changes in distribution or persistence of endotoxin. c) During the 12 weeks of the study, 60 to 90% of inoculated marker was retained by the mice. d) The liver retained the major part of the endotoxin. Next, in order, were spleen, lungs, and mesenteric lymph nodes.

4. Dr. Gander spent 15 weeks at RML with Dr. Rudbach studying immunological properties of the peptidogalactomannan (PGM) antigen of Penicillium charlesii. This polymer, of 150,000 mol. wt., contains about 10% amino acids and 14 polysaccharide side-chain residues, each with a terminal nonreducing galactofuranoside followed by 2 mannopyranosides. The object was to establish the relative immunological importance of chemical constituents of PGM, particularly the unique furanose form of galactose.

The assay consisted of a radioisotope precipitation test with ^{3}H-PGM, rabbit antiserum to PGM, and ammonium sulphate as the precipitant. When increasing molar ratios of various constituents and chemically altered preparations of PGM were added to the assay mixture, the reaction was increasingly inhibited and the slope was controlled by the strength of the inhibitor. Thus the immunochemical importance of the various groupings on PGM could be evaluated. The inhibitors tested were: PGM, peptidomannan (PM) from a mutant of P. charlesii, galactomannan obtained by beta-elimination from PGM, mannan obtained by beta-elimination from PM, galactose, mannose, methyl alpha- and beta-D-galactofuranoside and pyranoside, and the peptide backbone of the polymer. Conclusions were: a) the carbohydrate side chains contain most or all of the immuno-determinant groups of PGM; b) of the side-chain sugars, galactofuranose and mannose, in that order, contribute most to the specificity of the immuno-determinant group; c) under the conditions of these experiments, no antibodies were directed to the peptide moiety of PGM.

5. Dr. M. Niwa has resumed his valued collaboration and has been supplied with equipment for centrifugal chromatography (Centri-Chrom). Our combined studies indicate that the so-called "glycolipid" endotoxins from heptoseless mutant cultures contain extraneous lipids which may be responsible for the designation. With anhydrous organic solvents employed in both TLC and Centri-Chrom, some of the extraneous lipids migrate as glycolipids, but the endotoxic material stays at the point of origin.

6. Other collaborative investigations have been continued or newly initiated. Dr. Hausmann is interested in the role of endotoxin from oral bacteria in the pathogenesis of periodontoclasia and has developed a tissue culture system in which oral bacteria and their products stimulate bone resorption. All endotoxins from unrelated organisms we have supplied him have been markedly effective in his system. Collaboration with Dr. Anacker and Dr. Venneman on distinguishing specific and nonspecific factors in resistance to infection has continued, but slowly, for lack of an investigator to work full time on this demanding field. The same applies to investigation of *Brucella* endotoxin with Dr. Leong. An excellent radioactive endotoxin with specific activity of 3.64 μc/mg was prepared from *E. coli* for Dr. Wolff.

7. Early reports of endotoxin-like activities of natural and synthetic polynucleotides were necessarily suspect because of the possibility of contamination with bacterial products. To check these reports, we purchased supplies of the polymers of synthetic single-stranded adenylic, cytidylic, inosinic, and uridylic acids. None of these was pyrogenic in rabbits or lethal to chick embryos or actinomycin-D-treated mice. Under rigidly aseptic conditions we then prepared double-stranded nucleotides from various pairs, monitoring the reactions by ultraviolet absorption. Some of the products were then found to be active in the foregoing bioassays, and of these the combination polyinosinic-polycytidylic acid (Poly I·Poly C) was by far the most potent.

Significance to bio-medical research and the program of the Institute:

1. Many untoward symptoms of infection with invasive gram-negative bacteria result from the poisonous effects of endotoxin, as does death in some forms of shock. Elucidating the mechanism of tolerance to endotoxin has a bearing on survival in these diseased states. We have distinguished

two unrelated mechanisms which can produce the same overt signs of tolerance, and we have found that antibody to the polysaccharide (O) antigenic determinants is sufficient to protect mammals against the poisonous effects. These results controvert certain prevalent ideas about the nature of tolerance but fully support and extend the recent findings of S. E. Greisman and colleagues. The fact that antibody to O determinants can block toxicity does not imply that these determinants are themselves toxic. The evidence that they are not is overwhelming. Perhaps toxic particles are rapidly destroyed by opsonization before damage is extensive, but other possible mechanisms should be explored. It is entirely conceivable that mild anaphylaxis could abort endotoxin fever in animals with homologous circulating O antibodies, but evidence is lacking that this happens in the rabbit. Specific antibody also protects against the lethal effect of endotoxin in mice and in this case it is known that anaphylaxis must be avoided to demonstrate the protective effect.

2. Thiobacillus ferrooxidans is responsible for the production of most of the sulphuric acid and ferric precipitates that make acid mine drainage a major pollution problem in this country. Its cell wall is studied because this is the point of contact with an acidic environment and must hold an important key to the explanation of how the organism utilizes both soluble organic and insoluble inorganic substrates. It was initially surprising to learn that a major constituent of the cell wall is an endotoxic lipopolysaccharide (LPS) with obvious resemblances to that of parasitic and commensal bacteria of man, although similar substances have also been found in free-living nitrogen-fixing bacteria (Azotobacter species). A further surprise was that the most toxic material for mammals came from these bacteria when grown on inorganic iron. It is widely believed that endotoxin plays a determining role in the virulence of enteric gram-negative bacteria, but this is probably an accidental perversion of its normal service to the bacterial cell. Several relevant discoveries now permit us to study this more basic function of endotoxin. It is also of fundamental biological interest that changes in substrate so profoundly alter antigenic and chemical composition of LPS. Such changes can occur with biochemically deficient mutants, but that cannot be the mechanism here.

3. Earlier studies of the distribution and persistence of endotoxin have been relatively short-term. Evidence that products of endotoxin are excreted has also been presented but not quantitatively assessed. Our finding that up to 90% of the label can be retained for at least 12 weeks demonstrates the need for longer follow-up and indicates that the body's method of dealing with endotoxin is not simple excretion. These results also show that the cheaper and less tedious method of external labeling provides a sufficiently stable marker for most purposes. Such persistence may help to explain the extraordinary "immunological memory" to molecular amounts of LPS antigens, which has been described in earlier reports.

4. Products of Penicillium species are known to contain allergens responsible for atopic sensitivity. Many other allergens are glycopeptides, with particle size of 10,000 to 100,000 daltons, in which carbohydrates provide the immunodominant groups to which the specificity of the reagin is directed. Thus, the peptidogalactomannan (PGM) of P. charlesii fits the general pattern of known allergens and may well be the incitor and elicitor of Penicillium (including penicillin) allergies. If so, it is the best characterized of the important allergens. This work applies to endotoxin studies because it provides an approach for identifying determinants particularly associated with toxicity or hypersensitivity.

5. It is considered unlikely that polynucleotides have any purely chemical ingredients in common with bacterial lipopolysaccharides. The finding that synthetic RNA, after double-stranding in sterile buffer, acquired endotoxin-like properties is, therefore, the most concrete evidence in favor of our frequently reiterated hypothesis that endotoxicity may depend more upon structural conformation—shape and size— of particles than upon chemical analysis. Certainly RNA contains no lipid A.

Proposed course of project:

1. Dr. David Monner is expected to arrive as a senior postdoctoral fellow near the beginning of fiscal 1972. He will have completed two years of postdoctoral study in Sweden and will continue here with an extremely well-defined, well-thought-out problem. He seeks to prove "A correlation between structure and function in the lipopolysaccharide of the Escherichia coli cell wall" by isolating and chemically characterizing receptor sites for certain specific bacteriophages and relating them to the phenotypic property of ampicillin resistance and the mating function of the cell. His problem will utilize many of the skills and pieces of special equipment we can provide. At the same time, our group will be fortified, in an area of weakness, by addition of a man trained in biochemical genetics.

2. As indicated in Project 179, we intend to progress from morphological studies of F. tularensis to chemical and biological examination of nonendotoxic lipopolysaccharides from its cell wall. It is believed that, through comparison of this material with "semiendotoxins" from Thiobacillus and Brucella species and with potent endotoxins from enteric bacteria, we can, by elimination, identify those structures and chemical entities responsible for host reactivity.

3. Mr. Jim Cutler, a doctoral candidate in microbiology at Tulane University, has received an ASM President's Fellowship to do part of his research for dissertation with Dr. Milner. The problem is to isolate and characterize in a definitive manner the possibly endotoxic material that has frequently been described in the yeast-like pathogenic fungus, Candida albicans.

4. Dr. Wheat has sent 50 extracts and side fractions from cells of polysaccharide deficient mutants grown in various ways both here and in Durham. Through biological and chemical studies on the effect of milieu, we hope to reconcile conflicting reports and establish common characteristics of active preparations.

5. On request to Dr. Ribi by Dr. K. F. Meyer, we are joining in an effort to clarify the role of endotoxin in pathogenesis and prophylaxis of plague. A large shipment of cells of Pasteurella pestis, grown for this study by Cutter Laboratories, has been received, and fractionation and testing are in progress.

6. Definite commitments to other activities include: a) further collaboration with Dr. Niwa, particularly on application of Centri-Chrom to the analysis of diverse endotoxins; b) continued association with Drs. Vestal and Lundgren in the immunochemical analysis of fractions from Thiobacillus ferrooxidans; c) cooperation with Dr. Leong, who has received outside support for pursuing the investigation of Brucella endotoxins at RML in the summer of 1971; d) collaboration with Dr. Rudbach and Dr. Ward in an attempt to define conditions under which normal humoral factors sometimes inactivate endotoxin, but at other times seem to potentiate it.

7. In connection with continued application of Centri-Chrome to the refinement and characterization of endotoxin, synthetic polynucleotides will also be examined to determine whether this primarily physical method of analysis will indicate any similarity of structure between RNA and bacterial lipopolysaccharides.

Honors and Awards:

Dr. K. C. Milner

Invited discussant, Symposium on Endotoxins, Infectious Diseases Society of America, Chicago, October 17-18, 1970.

Reappointed Faculty Affiliate (Lecturer in Medical Microbiology), University of Montana, Missoula.

Publications:

Rudbach, J. A.: Some requisites in systems leading to hybrid formation between bacterial endotoxins. J. Infect. Dis. 122: 139-145, 1970.

Publications (Cont'd):

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Serial No. NIAID-179
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The role of morphological elements of microorganisms in immunity and related phenomena

Previous Serial Number: Same

Principal Investigator: E. Ribi

Other Investigators: R. L. Anacker, W. D. Bickel, W. R. Brown, G. Goode, S. C. Harris, K. C. Milner, and R. F. Smith

Cooperating Units:
Ivan Sorvall, Inc., Hamilton, Montana: Mr. C. J. Filz and Dr. D. G. Petcoff
Dr. K. Yamamoto, Hokkaido University, Sapporo, Japan
Mr. S. M. Strain, MIT, Cambridge, Mass.
Dr. R. W. Walker, University of Massachusetts, Amherst

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 6/12			3 6/12
Professional:	2 5/12			2 5/12
Other:	1 1/12			1 1/12

Project Description:

Objectives:

Studies of the fine structure and chemical composition of microorganisms and the correlation of these attributes with certain biologic properties of cells or cell fractions, and the development of new tools and techniques and their application to current problems comprise the activities of this section. This year's studies have been concerned chiefly with the submicroscopic morphology of endotoxin and extending the application of centrifugal microparticulate bed chromatography to the separation of certain sugars and the isolation and quantitation of the important cannabinoids of marihuana and hashish.

Methods employed:

A combination of biophysical and biochemical techniques has been employed. Problems requiring new methodology or specialized uses of the electron microscope, Sorvall cell fractionator, and centrifugal micro-particulate bed chromatography have been assigned to this project.

Major findings:

1. It has been an attractive hypothesis, first proposed by RML investigators, that the structural organization of bacterial endotoxins might provide the key to understanding their mode of action. In further exploration of this idea, we have returned this year to the study of morphology with the aid of electron microscopy. It seemed possible that one of the obstacles to resolution of structure might be the heterogeneous nature of ordinary preparations. Therefore, we decided to employ the most highly refined endotoxins available.

An elaborately refined, highly toxic, aqueous ether extract of Salmonella enteritidis was found to contain only 2.2% total fatty acid and to be protein- and peptide-free. When dried films were stained negatively with potassium phosphotungstate, we observed ribbon-like structures about 200 Å wide and of various lengths from short rectangles to long branching filaments. Flat disks of about 500 Å in diameter, which were free of any details of fine structure, were also observed. Infrequently, the rectangular particles and shorter ribbons contained an unstained core, running the entire length of the particles, surrounded by stained areas which could have been interpreted as deposits of stain between fine side chains extended laterally from a dense core structure. In similar films stained positively with uranyl acetate, there were the same structureless ribbons and round or oval flats. Infrequently, the short rectangles showed a double-line structure of parallel densely stained areas with overall width of about 100 Å.

2. Another approach we thought might be revealing was examining the morphology of lipopolysaccharide-containing materials that were not endotoxic. Well over a decade ago we had found that treatment of whole cells of Francisella tularensis with aqueous ether caused rupture of the cells. This unusual fragility is now thought to result from a deficiency of the rigid peptidoglycan layer possessed by most gram-negative bacteria. The sedimentable cell wall material, which may be regarded as a lipopolysaccharide-protein complex, contains the major serologic and immunogenic activities of the whole cells but is lacking in ability to stimulate most of the pathophysiologic responses associated with endotoxin.

This method of preparation can hardly be termed an extraction, and the crude product probably contains most of the particulate matter of the cell envelope; yet we observed that preparations from different cultures of F. tularensis, stained positively or negatively, as above, or shadow-cast with metals, reproduced most of the morphological structures which have been described for extracted endotoxins, and in roughly the same order of apparent size. For reasons not yet understood, however, more details of fine structure could be resolved in these preparations than in films or stained sections of extracted endotoxin. Thus, many rectangular, disk, or doughnut forms could be seen to be collapsed spheroids, often with wrinkled or curled edges that gave the appearance of parallel dense lines, as seen in endotoxins. Some strains yielded predominantly round flats (collapsed spheroids); others gave mainly filamentous structures, and some a mixture of each. The filaments were clearly not single-thickness ribbons, but collapsed tubules or rolls of membranes, some of which could be seen extruding from spheroids.

3. As in the previous year, continued development and application of centrifugally accelerated chromatography on gels prepared with ultrafine silica (Centri-Chrom) have been emphasized. The method has been extended to include resolution and identification of important components in marihuana and hashish. Rapid qualitative analysis was achieved. The samples were extracted with petroleum ether, the extraction products were dissolved in cyclohexane and the resulting solutions were then chromatographed on gel prepared with microparticulate silica and petroleum ether-diethylamine (99:1v/v). This same solvent system served as the mobile phase. Various adjustments of technique and numerous solvent systems were tested in order to determine the most suitable combination. Separation of the following components in both artificial mixtures of synthetic standards and extracts of marihuana and hashish was achieved: cannabichromene, cannabinol, Δ^9-tetrahydrocannabinol, Δ^8-tetrahydrocannabinol, cannabidiol and cannabicyclol. The chromatograms were sprayed with di-o-anisidine tetrazolium chloride reagent (Echtblausalz) to produce specific color reactions in the bands formed by the compounds. The migration rates were calculated as the ratio of distance of migration to the length of the chromatogram (D/L). These values were 0.11, 0.22, 0.52, 0.60, 0.76 and 0.91, respectively. They were reproducible within 0.01 and demonstrate that the difficulties in adequately separating certain of these standards by thin-layer and gas-liquid chromatography have been overcome.

Comparison of the width and color intensity of each of the bands in the chromatograms of the extracts with the corresponding bands produced by known amounts of the synthetic standards permitted reliable estimation of important cannabinoids in samples of marihuana and hashish. Analysis of a sample and estimation of the amounts of its major cannabinoids was performed in about one-half hour. By our estimates "street" samples of hashish contained 0.5 to 4 percent Δ^9-tetrahydrocannabinol, the physiologically active component.

To determine the fate of Δ^9-tetrahydrocannabinol during the smoking process, a smoking machine was constructed and the synthetic standard was smoked. The smoke condensate was estimated to contain 60 percent unaltered Δ^9-tetrahydrocannabinol and 40 percent cannabinol. Combined, these components represented about 50 percent of the standard before it was smoked; thus, about one-half of the Δ^9-tetrahydrocannabinol was destroyed in other ways by burning. A similar increase in cannabinol content at the expense of Δ^9-tetrahydrocannabinol was observed when marihuana samples were machine-smoked. The absence of Δ^8-tetrahydrocannabinol in our chromatograms of smoke condensates supports recent findings by others that isomerization of Δ^9-tetrahydrocannabinol into Δ^8-tetrahydrocannabinol, another physiologically active component, occurs only to a very small extent, if at all, under smoking conditions.

Extracts of thyme, oregano, sage, rosemary, clove, caraway seeds, alfalfa, tobacco, green tea and rhubarb leaves were chromatographed, because these substances reportedly have been used to dilute or substitute for marihuana, or have been confused in other methods of analysis. Thyme and oregano each formed a single band with a D/L value of 0.34, identified as thymol. Clove formed an unidentified single band, which migrated at a slightly faster rate (D/L 0.35); the other extracts did not produce detectable bands. In no case did extracts from these purported diluents or substitutes mask the bands of cannabinoids, alter migration, or interfere with identification, when mixed with the synthetic standards.

4. The technique of centrifugal chromatography employing columns of microparticulate silica has been successfully applied to the qualitative separation of various sugars. Not only can monosaccharides, disaccharides, and trisaccharides by distinguished from one another, but, in addition, most sugars within these classes so far examined can also be separated from each other. Sugars in certain complex mixtures were completely resolved in 13 minutes; less time would be necessary for simpler mixtures. Five-μg samples of the sugars were detected upon heating of the columns sprayed with sulfuric acid-dichromate reagent. Aminosugars were identified with ninhydrin. Several of the sugars migrate at the same rate in the solvent systems used so far; however, after additional experimentation, systems which will permit separation of all sugars probably will be developed.

5. Last year Dr. Robert W. Walker performed preliminary experiments, results of which indicated that centrifugal microparticulate bed chromatography might be useful for the separation and identification of triglycerides. This work has been continued and the presence of C_{48}, C_{54}, C_{60} and C_{66} triglycerides in extracts of Mycobacterium smegmatis and M. bovis was substantiated. These findings have been published in Lipids vol. 5, 684-691, 1970.

Significance to biomedical research and the program of the Institute:

The various structures seen in cell wall extracts bear on the interpretation of electron micrographs of endotoxin and the deductions regarding structure that may be warranted. The use of a highly refined preparation (we avoid the word "purified" because we doubt that purity has been achieved) may have given somewhat improved resolution, but the forms observed did not differ essentially from those seen in crude products. In fact, the whole cell envelope fraction of an organism free of endotoxin contained structures indistinguishable from those seen in endotoxic material. This finding suggests that studies of the submicroscopic morphology of endotoxin will not lead directly to conclusions about its mode of action.

Increasingly, the structure of endotoxins, like that of biological membranes in general, is being interpreted in terms of an adapted Davson-Danielli model of a bimolecular leaflet. In this conception, the hydrophobic lipid ends line up in apposition with the hydrophilic polysaccharide chains extended to the outside. Our results with endotoxin may be interpreted by this model, but the observations on F. tularensis signal caution. In both cases, it seems to us unlikely that the occasional appearance of parallel dense lines in positively-stained preparations are instances of bimolecular leaflets seen edge-on. Why should leaflets stand on end only in positively stained preparations?

In view of the need for a simple, rapid method for the separation and identification of cannabinoids in Cannabis sativa, centrifugal microparticulate bed chromatography was applied for this purpose. It provided high sensitivity and resolution and at the same time permitted estimation of the concentration of cannabinoid components, in particular the major physiologically active Δ^9THC in marihuana and hashish samples. The technique also meets a need for an improved analytical method for both the chemist and biologist involved in marihuana research.

Centrifugal chromatography can also greatly speed analysis of biological samples for constituent sugars. Thin-layer and paper chromatographic procedures generally require several hours or more for completion.

Proposed course of project:

In view of the similarity of the physical structures of the lipopolysaccharidic somatic antigens of both the nontoxic F. tularensis and the toxic Enterobacteriaceae, we intend to determine whether chemical analysis reveals either qualitative or quantitative differences that could be responsible for toxicity, in particular whether the toxicity may be due to a very minor component present in one and absent in the other. Efforts will be made to improve the quantitation of components in chromatograms of cannabinoids by better statistical methods for evaluating the visual estimated and by mechanical scanning of the chromatograms. Work is in progress to extend the application of the technique to the separation of cannabinoid acids. The method is expected to be useful for this purpose because of the short time required for an analysis of these labile acids.

Honors and Awards:

Dr. E. Ribi

Continued as member, Tuberculosis Panel of the U.S.-JAPAN Cooperative Medical Science Program.

By invitation, presented seminar in Department of Microbiology and Public Health, Michigan State University, June 1970.

By invitation, presented a paper at Symposium on Chromatographic Methods at annual meeting of Am. Chem. Soc. and Can. Chem. Soc., Toronto, Canada, June 1970.

Served as chairman and presented paper at Sixth Annual Tuberculosis Research Conference sponsored by the U.S.-Japan Cooperative Medical Science Program, Tokyo, Japan, August 1970.

By invitation, presented lecture at the annual meeting of the Infectious Disease Society of America, Chicago, October 1970.

Served as consultant at Conference on Neisseria Biology, sponsored by NIAID, Bethesda, May 1971.

Publications:

Ribi, E., Filz, C. J., Goode, G., Strain, S. M., Yamamoto, K., Harris, S. C., and Simmons, J. H.: Chromatographic separation of steroid hormones by centrifugation through columns of microparticulate silica. J. Chromatogr. Sci. 8: 577-580, 1970.

Serial No. NIAID-180
1. Rocky Mountain Laboratory
2. Serological Technology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The encephalitides

Previous Serial Number: Same

Principal Investigator: L. A. Thomas

Other Investigators: C. E. Yunker

Cooperating Units: Dr. Michael Goloff, (EIS) Montana State Department of Health, Helena
Dr. R. B. Mackenzie, Rockefeller Foundation, Bogota, Colombia
Mr. J. Stout, Department of Zoology, Washington State University, Pullman

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	10/12			10/12
Professional:	3/12			3/12
Other:	7/12			7/12

Project Description:

Objectives:

Major objectives of this project are: 1) to obtain further information regarding viremia and antibody response of garter snakes following experimental inoculation with western equine encephalitis (WEE) virus—thus providing a better understanding of the possible role of these cold-blooded vertebrates in the ecology of this disease, 2) to determine prevalence of antibodies to certain arboviruses in selected human and animal sera submitted to this laboratory for special study, and 3) to characterize and determine antigenic relationship of viruses isolated from field material—special emphasis being placed on viruses isolated or transmitted by ticks.

Methods employed:

Standard procedures are used in all phases of the study, including preparation of antigens and immune sera, isolation and identification of viruses and serological tests (plaque-reduction, hemagglutination, hemagglutination-inhibition (HAI), complement-fixation (CF), and neutralization).

Major findings:

1. Viremia in WEE-virus experimentally infected garter snakes at various times before and after holding at 4°C. Garter snakes without WEE viremia or antibodies were inoculated intraperitoneally (IP) with 316,000 suckling mouse IP LD_{50} of WEE virus on 12/15/69. After incubation for 7, 11, or 35 days at 22°C and 4-5 months at 4°C, they were removed from the cold room and held at 22°C for the duration of the study. Uninoculated "normal" snakes were included as controls in each group. Blood was obtained at various times and tested for viremia in "wet" chicks or suckling mice and for neutralizing antibodies in adult mice. Some sera were tested for plaque-reduction properties. A few snakes received second and third inoculations of WEE virus (approximately the same as the first dose) on 9/14/70 and 11/10/70. All snakes were exsanguinated on 12/14/70.

Viremia was detected in only 50% of the snakes after IP inoculation of WEE virus. This finding is in contrast to previous results when most locally-collected snakes developed viremia after bite by infected mosquitoes or inoculation of stock virus. The difference in susceptibility might be attributed to such factors as seasonal variations in physiology of the snakes, presence of WEE antibodies not detectable by usual testing methods, or interference in multiplication of virus by an unknown inhibitor. Antibody titers resulting from a vigorous challenge were so low that one wonders whether the HAI and neutralization tests are satisfactory methods for determining past exposure of snakes to WEE virus. However, there was good correlation between HAI and neutralization tests even though titers were low. Results of plaque-reduction tests obtained thus far do not correlate either with viremia or HAI and neutralization test results. Additional plaque-reduction tests are scheduled. It appears that viremia does not recur in snakes after reinoculation of WEE virus whether or not antibodies are demonstrable.

2. Arbovirus activity in the region. In collaboration with the Montana State Department of Health we received mosquitoes taken during July and August from eastern Montana for tests for viruses. A total of 1270 non-*Culex tarsalis* and 4 *C. tarsalis*, comprising 30 pools, were negative for virus when tested in suckling mice. Most mosquitoes were dead upon arrival. However, our results were similar to those of CDC investigators, Fort Collins, Colorado, who also collected mosquitoes during

this time in eastern Montana. They isolated WEE once from 2631 *C. tarsalis* tested. None of 22 sentinel chickens exposed to mosquitoes during June and July developed WEE HAI antibodies and only 2 developed St. Louis encephalitis (SLE) antibodies. These results support other evidence that WEE virus activity was low in the area in 1970.

Surveillance of arbovirus activity was also achieved by testing sera submitted from patients thought to have viral encephalitis. Eighty human and equine sera from Montana and 19 from South Dakota were tested for HAI and/or CF antibodies against WEE, SLE, and California encephalitis viruses. A serviceman, who became ill shortly after returning from overseas, had a broad group B antibody response with the highest CF titer against West Nile virus. Other serologic findings suggested little local virus activity. HAI antibodies against SLE were detected in only 2 patients and HAI antibodies against WEE were detected in only 4 patients and 2 horses.

Significance to bio-medical research and the program of the Institute:

The role of cold-blooded vertebrates in the ecology of WEE virus is not settled. Studies the past year have contributed information regarding viremia patterns and antibody response in relationship to temperature and repeated inoculation of virus. These data will be useful for evaluating results of others working on this problem. Study of mosquito-borne viruses complements work on tick-borne viruses particularly from the standpoint of strain identification.

Proposed course of project:

Most of our attention will involve tick-borne viruses. Additional strains of "known" tick-borne and possibly mosquito-borne viruses will be added to our working collection for characterizing field isolates.

Efforts to prepare a satisfactory California HA antigen will be continued. Suckling mouse brain will be used for cultivation of additional strains of California virus.

WEE virus in snakes will not be studied as extensively as it has been in the past, but we will compare the response of snakes collected from the Bear River Refuge, Brigham City, Utah, to those collected in Montana.

We will continue our surveillance of sera referred from outside agencies for antibodies against selected arboviruses.

Serial No. NIAID-181
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tick-borne viral disease agents

Previous Serial Number: Same

Principal Investigators: C. M. Clifford, C. E. Yunker, and L. A. Thomas

Other Investigators: W. Burgdorfer, L. E. Hughes, and J. E. Keirans

Cooperating Units: Mr. J. Bushman, Deseret Test Center, Ft. Douglas, Utah
Dr. J. E. George, Texas Technological College, Lubbock
Dr. A. Greichus, South Dakota State Univ., Brookings
Mr. R. Gresbrink, Oregon State Board of Health, Portland
Dr. H. Johnson, Rockefeller Foundation, Viral and Rickettsial Dis. Lab., Univ. of Calif., Berkeley
Oregon State University, Corvallis: Mr. E. Easton and Dr. R. Goulding
Washington State University, Pullman: Dr. V. Schultz and Mr. J. Stout

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	5 2/12			5 2/12
Professional:	1 10/12			1 10/12
Other:	3 4/12			3 4/12

Project Description:

Objectives:

To determine the incidence, distribution and ecology of tick-borne diseases that influence man and domestic animals in the United States. Of equal importance is the investigation of the vector relationships of the increasing number of viruses known or thought to be associated with ticks.

Methods employed:

Field and laboratory approaches include conventional and specialized entomological, ecological, virological and immunological techniques.

Major findings:

1. Viruses and ixodid ticks. Since the last report, 289 and 266 pools of ticks from Oregon were tested in suckling mice and African green monkey-kidney cell line (Vero) cells, respectively. Fourteen isolates of Colorado tick fever (CTF) were recovered and these were confirmed by mouse and plaque-reduction neutralization tests.

In collaborative studies with Mr. Jack Stout, Department of Zoology, Washington State University, we tested 345 small mammal serums for CF antibodies against Western equine encephalitis, CTF, Powassan, Modoc, and Rocky Mountain spotted fever (RMSF) antigens. Antibodies were detected in 2 serums: a titer of 1:8 against RMSF in one and a titer of 1:8 against Modoc virus in the other serum.

Last year we reported the unusual occurrence of *Dermacentor variabilis* (the American dog tick) from an area 12 miles northwest of Pullman, Washington, which is at least 800 miles from its previously known distribution. A short survey in this region revealed that this species is widespread throughout the Lewiston, Idaho,-Pullman, Washington, area. All tests on *D. variabilis* for the presence of CTF virus and RMSF rickettsia were negative.

Studies were continued to establish the identity of the group B virus isolated from *Ixodes uriae* collected on Three Arch Rocks Wildlife Refuge near Tillamook, Oregon. Virus stock, antigen and immune serums were sent to Dr. Casals, Yale Arbovirus Research Unit, and he demonstrated by CF and mouse neutralization tests that this isolate was identical to Tuleniy virus isolated recently by Dr. Lvov from *I. uriae* collected on islands off the eastern coast of Russia.

Another trip was made to the Oregon Coast during 1970 and a much larger sample of *I. uriae* was collected from Three Arch Rocks. This same species of tick was found in large numbers on a Yaquima head near Newport, Oregon. These ticks were segregated into 100 pools of 10 ticks each and injected into suckling mice and Vero cells. Forty of 100 and 14 of 97 pools of *I. uriae* tested in suckling mice and Vero cells, respectively, yielded virus. From these, 5 strains that displayed different characteristics in mice and tissue culture were selected as "prototype" for more intensive study. Mouse brain antigens have been tested against these 5 prototype immune serums and ascitic fluids, and by the CF test, one of the prototype viruses has proven to be Tuleniy virus. Some of the other agents appear to be related to Kemerovo-Chenuda group viruses.

The 10 isolates made in Vero cells have been plaque-purified and an additional 4 agents were recognized and separated during these procedures. Through plaque-reduction neutralization tests against selected antiserums these isolates appeared to belong to 4 different serological groups.

The agents recovered in mice have been passaged to increase their titer and are currently being compared with the "prototype" strains in mouse-neutralization tests. Preliminary results have disclosed that 2 are Tuleniy virus, but several other, as yet, undetermined viruses are present. Approximately 25 antigens have been prepared from brain tissue of suckling mice infected with these unidentified tick viruses.

In conjunction with these studies 34 cormorant and pelican serums, collected in South Dakota by Dr. Greichus, were tested for hemagglutination-inhibiting antibodies against Three Arch Rocks (Tuleniy), Jap B (N), and West Nile viruses. There was no indication that these birds had been infected with a group B virus. However, in tests of serums from seabirds from the Central Pacific titers of 1:10 to 1:40> against group B viruses were demonstrated in 25/212 serums; this finding suggests past exposure to a group B virus or viruses.

2. Duration of association of CTF virus with red blood cells in mammals. Emmons recently demonstrated, in some preliminary studies, that CTF virus would remain in red blood cells of patients long after it was undetectable in whole blood samples, because antibody neutralized the virus in conventional testing procedures. To substantiate these preliminary findings we used the washed cell technique of Emmons to test for this virus in red blood cells of 8 patients with confirmed CTF. Virus was present in all patients sampled for 90 days. For the first 50 days the titers ranged from 2.0 to 4.3. Afterwards, the amount of virus gradually decreased until 90 days when titers of virus ranged from 0.1 to 1.7. Virus could still be detected at 110 days, and one sample was still positive at 120 days. Currently, experiments in rats and mice have shown the virus (titer 2.7) to be present at 45 days. The actual duration of viremia will be determined as the experiment progresses.

3. Viruses and argasid ticks. The prototype strain of Sixgun City virus, originally isolated in Vero cells from *Argas cooleyi* ticks (see last Annual Report) could not be passed in suckling mice, and the virus content of tissue culture material was inadequate for satisfactory CF reagents. By alternating passage of this virus between suckling mice and Vero cells,

without intervening freezing, this strain was adapted to mouse brain, from which satisfactory CF antigen could be made. At present, results of CF, mouse and plaque-reduction neutralization tests indicate that Sixgun City virus is a strain of Mono Lake (from Argas ticks in California) virus and is closely related to Huacho (from Ornithodoros ticks in Peru) virus. The mouse brain stock prepared from the "prototype" strain has allowed us to confirm that this isolate is identical to those originally isolated in suckling mice. In addition, Sixgun City virus has been isolated from A. cooleyi collected in Colorado, thereby extending its distribution in the U.S.

Further work has been completed on isolates other than Sixgun City reported in the previous report. Results of CF, mouse and plaque-reduction neutralization tests have shown that 2 are closely related to Punta Salinas (from Ornithodoros ticks in Peru) and Sapphire II (from Argas ticks, Granite Co., Montana).

4. Assay systems for tick-borne viruses. Development of assay systems for tick-borne and related viruses in mammalian cell cultures has been continued. Since last report, 29 viruses were shown capable of causing cytopathogenic effect (CPE) or plaques in Vero, baby hamster kidney (BHK 21 and BHK 0853), or porcine kidney (PK-H13) cells. Vero cells were most susceptible to the tick-borne viruses and responded with CPE when infected with Wad Medani, Silverwater, Quaranfil, Lone Star, Chenuda, and Kemerovo (R-10) viruses. Non-tick-borne entities tested for comparison and responding similarly were Marituba, Gulu, Sandfly fever (Naples), Montana myotis leucoencephalitis (MML), pseudorabies, encephalomyocarditis, and mouse encephalomyelitis (GD-7 and FA 660). Seventeen other viruses, 3 of which were the tick-borne agents, Powassan, Langat and Chenuda, failed to cause CPE in Vero cells. Viruses causing plaques in Vero cell monolayers were: Matucare, Wad Medani, Sawgrass, Quaranfil, Silverwater, Kemerovo, Chenuda, West Nile, Turlock, California (La Crosse), Tahnya, Rio Bravo, Chikungunya, Marituba, Gulu, Sandfly fever (Naples), MML, pseudorabies, and FA 660. Failing to produce plaques in Vero cells were 10 other viruses, including the tick-borne agents, Tuleniy (Three Arch), Lone Star, Powassan and Langat. Many of the preceding viruses also failed to cause CPE or plaques in BHK 21, BHK 0853 and PK-H13 cells. However, Tuleniy (Three Arch), Powassan, and Langat, 3 tick-borne members of Casals' serological group B, which produce no gross signs in Vero cells, could be detected by CPE or plaque production in BHK 21 cells.

Significance to bio-medical research and the program of the Institute:

As more viruses associated with ixodid and argasid ticks are being discovered, some interesting relationships have developed. The group B agent isolated from the Oregon Coast (Three Arch Rocks virus) has been shown to be conspecific with Tuleniy virus isolated from the same species of tick off the eastern coast of Russia. These findings suggest that group B arboviruses associated with seabirds may have a wide distribution. Furthermore, the vast numbers of hosts in rookeries and the tremendous numbers of ixodid and argasid ticks associated with them produce an effective means for the circulation of various disease agents. Of additional interest is the number of different viruses that have been found in a single location. Preliminary results on identification of some of these agents indicate that some of these agents are related to the Kemerovo-Chenuda group viruses. This fact again suggests that there may be a movement of virus from seabirds to species that live further inland. Evidence of the occurrence of this phenomenon has been amply furnished by our studies on viruses associated with argasid ticks in Texas, Colorado and Montana. In two instances viruses isolated from A. cooleyi, that feeds exclusively on inland birds, have been shown to be closely related and probably identical to agents isolated from ticks that feed only on seabirds.

It has been postulated that these group B viruses associated with seabird ticks may be the evolutionary precursors of the group B agents like Jap B and West Nile virus that are associated with mosquitoes and cause extensive human illness in various parts of the world. It has also been suggested that these viruses may overwinter in ticks and are made available to mosquitoes during the spring nesting season when the ticks are parasitizing the birds.

The development of tissue culture assay systems for arboviruses amplifies the means available for recognition, identification and differentiation of these viruses.

Studies on the longevity of the association of the CTF viruses with red blood cells in humans and rodents may give us a logical explanation for the overwintering of this virus which would explain why it is so successfully maintained in certain areas of the West.

Proposed course of project:

Studies on the distribution and incidence of tick-borne viruses in the Northwest will continue, again, mainly through collaborative efforts with the Oregon State Health Department, and Oregon State and Washington State Universities.

Isolation and characterization of viruses associated with ticks will continue. Emphasis will be placed on the antigenic comparison of viruses already on hand.

Because of the current belief that these newly isolated group B viruses are actually mosquito-borne viruses being overwintered or maintained in ticks we plan to attempt some transmission studies. Our tissue culture studies to date indicate that these viruses will not grow in mosquito cell lines, but will in tick explants.

Work will be continued to determine how long CTF virus will remain in red blood cells of various mammals.

Publications:

Yunker, C. E.: Worldwide research on human and animal diseases caused by tickborne viruses. Misc. Publ. Entom. Soc. Amer. 6: 330-338, 1970.

Clark, G. M., Clifford, C. M., Fadness, L., and Jones, E. K.: Contributions to the ecology of Colorado tick fever virus. J. Med. Entom. 7: 189-197, 1970.

Burgdorfer, W. and Mavros, A. J.: Susceptibility of various species of rodents to the relapsing fever spirochete, Borrelia hermsii. Infect. Immun. 2: 256-259, 1970.

Clifford, C. M., Yunker, C. E., Easton, E. R., and Keirans, J. E.: Ectoparasites and other arthropods from coastal Oregon. J. Med. Entom. 7: 438-445, 1970.

In press:

Clifford, C. M., Yunker, C. E., Thomas, L. A., Easton, E. R., and Corwin, D.: Isolation of a group B arbovirus from Ixodes uriae collected on Three Arch Rocks National Refuge, Oregon. Amer. J. Trop. Med.

Serial No. NIAID-182
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The epidemiology of human infections of special interest

Previous Serial Number: Same

Principal Investigator: R. N. Philip

Other Investigators: E. A. Casper, R. K. Gerloff, and L. A. Thomas

Cooperating Units: Dr. F. B. Gordon, Naval Medical Research Institute, Bethesda, Maryland
Dr. A. B. Greaves, Northwest Central Venereal Disease Clinic, Department of Public Health, Washington, D. C.
Dr. D. A. Hill, LID, NIAID, NIH, Bethesda, Maryland
Indian Health Service, Billings Area, Billings, Montana

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	7/12			7/12
Professional:	2/12			2/12
Other:	5/12			5/12

Project Description:

Objectives:

Studies in this project acquire basic information about the epidemiology and prophylaxis of human infections of special interest to RML.

Methods employed:

Field investigations coordinated with diagnostic services by the Epidemiology and Serological Technology Sections are emphasized. The methods are standard epidemiologic, statistical and diagnostic laboratory procedures. As the situation warrants, specialized laboratory techniques are used for diagnosis.

Major findings, significance to bio-medical research and the program of the Institute, and proposed course of project:

This project was inactive during the year. Work on chlamydiae was confined to laboratory studies (*see* Project NIAID-185) with the anticipation that the findings derived therefrom will be applicable to reactivation of this project. Specifically, a means for serotyping of strains by micro-immunofluorescence should help to define the epidemiology of TRIC eye infections among Montana Indians and the etiologic relationship to infection of the genital tract. Negotiations are presently under way with the Indian Health Service to obtain isolates from both anatomical sites for comparison.

Honors and Awards:

Miss Elizabeth Casper

Served as Secretary of the Montana Health Association.

Dr. Robert Philip

Member of the Executive Committee, Montana Health Association.

Publications:

In press:

Philip, R. N., Hill, D. A., Greaves, A. B., Gordon, F. B., Quan, A. L., Gerloff, R. K., and Thomas, L. A.: Study of chlamydiae in patients with lymphogranuloma venereum and urethritis attending a venereal diseases clinic. *Brit. J. Vener. Dis.*

Hill, D. A., Philip, R. N., Greaves, A. B., and Purcell, R. H.: Study of mycoplasmas in patients with urethritis attending a venereal diseases clinic. *Brit. J. Vener. Dis.*

Serial No. NIAID-183
1. Rocky Mountain Laboratory
2. Comparative Pathology and Arbo and Chronic Viral Diseases Sections
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Viruses as a probable cause of certain chronic diseases of man

Previous Serial Number: Same

Principal Investigator: W. J. Hadlow

Other Investigators: R. K. Bergman, J. E. Coe, R. C. Kennedy, G. C. Lavelle, D. L. Lodmell, and R. E. Race

Cooperating Units: Animal Health Division, Agricultural Research Service, U.S.D.A.: Dr. A. L. Klingsporn, Hyattsville, Md., and Mission, Texas
Dr. K. K. Takemoto, LVD, NIAID, NIH, Bethesda, Md.
Dr. C. M. Eklund, Minneapolis, Minnesota

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	19 1/12			19 1/12
Professional:	5 2/12			5 2/12
Other:	13 11/12			13 11/12

Project Description:

Objectives:

From a study of naturally occurring slow viral diseases of domestic animals, 1) to obtain an understanding of the mechanisms by which viruses cause chronic diseases and 2) to develop working concepts and investigational procedures that will facilitate determining whether viruses are causally related to some chronic diseases of man.

Methods employed:

Four diseases that represent the models for continued study include: 1) scrapie, a subacute or chronic encephalopathy of sheep and goats, 2) a comparable neurologic disease of ranch mink, 3) Aleutian disease, a protracted systemic disease of mink, and 4) progressive pneumonia, a chronic proliferative pulmonary disease of sheep. Clinicopathologic, virologic, and immunologic features are studied in an effort to gain an understanding of the natural history and pathogenesis of each disease. Characterization of the causative viruses is of secondary concern, and is pursued within the limits imposed by the availability of personnel competent to do this.

Major findings:

1. Scrapie

a. The disease process. Titration of tissues from 9 sheep affected with natural scrapie was continued, but little can be added to that mentioned last year.

The spleen plays an important role in the pathogenesis of scrapie virus infection in the mouse. For example, virus appears early and reaches high concentration in this organ. As mentioned last year, an effort was made to associate scrapie virus infectivity with particular splenic cell populations. Accordingly, splenic cells from mice inoculated with scrapie virus were separated into subpopulations by differential flotation in density gradients of isotonic albumin. When these subpopulations were assayed in mice for virus, several were found to have a 50- to 100- fold "enrichment" of virus-infected cells as compared to the cells/LD_{50} value for the whole spleen-cell suspension. Cells richest in infectivity were generally found in the less-dense cell fractions. Apart from providing some clue about the site of viral replication in the spleen, these results indicate that the separational procedure used should be useful in efforts to prepare highly infectious cell populations for other studies.

b. The virus. The viruses of scrapie and mink encephalopathy have several properties in common and at present can be distinguished only by differences in experimental host susceptibility. To determine the susceptibility of mink to scrapie virus, 9 pastel mink were inoculated intraperitoneally with $6 \times 10^{6.48}$ mouse LD_{50} of mouse-adapted scrapie virus (Chandler strain). All remained clinically normal for 4 1/2 years and were then challenged intracerebrally with 10^3 or 10^4 LD_{50} of encephalopathy virus. All became affected with typical encephalopathy 6 to 7 months later. Thus, a large amount of mouse-adapted scrapie virus failed to cause disease in these mink or to protect them against infection with the closely related encephalopathy virus. Because the scrapie virus used and its route of injection may not have provided an adequate test, 3 groups of 4 pastel mink which were inoculated intracerebrally with brain suspensions of known high virus titer from 3 sheep affected with natural scrapie. Results of this experiment will not be available for at least another 6 months.

Here and elsewhere, ether treatment of scrapie virus was found to reduce its infectivity 1 to 2 $\log_{10}$, that is, 90 to 99%. If this effect of ether is due largely to its lipid solvent properties, then chloroform, a more efficient lipid solvent, should have a greater effect on infectivity than ether, as it does on known lipid-containing viruses. However, chloroform treatment resulted in about 1 $\log_{10}$ less loss of infectivity than did treatment with ether. This result may indicate an unusual effect of ether on scrapie virus or other constituents necessary for infectivity, and may provide an insight into the nature of the virus.

2. Encephalopathy of mink

a. The disease process. Nothing new to report.

b. The virus. As already mentioned, the relationship of encephalopathy virus and scrapie virus is still uncertain. The failure of encephalopathy virus to cause a scrapie-like disease in mice has been regarded by us as the one important distinguishing property, especially in view of the ease with which we can isolate wild scrapie virus in mice. Moreover, others have reported only equivocal results in similarly inoculated goats. This distinction based on differences in experimental host range now seems less valid. For example, between 29 and 33 months after they were inoculated intracerebrally with encephalopathy virus (Idaho isolate), 7 of 19 goats became affected with a slowly progressive neurologic disease resembling scrapie. Initial hyperexcitability, tremor, incoordination of gait, visual impairment, and terminal somnolence and prostration were the main clinical signs observed over a steadily debilitating course of 1 to 4 months. Histologic changes in the brain, chiefly astrocytosis, neuronal degeneration (including vacuolation), and spongiform change in gray matter, were essentially like those observed in goats affected with experimental scrapie. The only significant departure from the topographic neuroanatomic distribution of the lesion in scrapie was the fairly regular occurrence of spongiform change in the cerebral cortex.

By contrast, during the same period of observation all of 19 Cheviot sheep remained clinically normal.

3. Aleutian disease (AD)

a. The disease process. In the study mentioned last year on the temporal distribution of virus in infected sapphire mink, the only new finding was the consistently high titer (10^{-4} to 10^{-7}) of virus detected in the intestine. The pathogenetic significance of this observation will require further study.

To obtain more information on the pathogenesis of AD in pastel mink, 20 of them were inoculated subcutaneously with 2 X 10^4 sapphire LD_{50} of AD virus. This amount of virus was calculated to produce disease in about half of the group. Beginning at 2 weeks after inoculation, the mink were examined for the presence of viremia and changes in serum gamma globulin levels. Only preliminary observations are available now.

As detected by inoculating sapphire mink intraperitoneally with 0.5 ml of a 10^{-1} suspension of whole blood, about half of the pastels had viremia at 2 and 4 weeks. Thereafter, only 5 continued to have detectable virus in the blood. One became affected with terminal AD at 10 weeks. The other 4 have remained clinically normal through 24 weeks, though their serum gamma globulin levels are moderately elevated. Most mink in which viremia occurred only at 2 and 4 weeks did not have any elevated serum gamma globulin through 24 weeks. In a few mink, however, the level of gamma globulin increased slightly, but remained stationary. Six mink never had viremia or elevations in serum gamma globulin. These results confirm an earlier observation that some mink of the pastel population studied here are completely refractory to overt infection with AD virus. Whether they eliminate all virus shortly after exposure is not known. At various intervals these negative mink will be examined for virus in spleen and mesenteric node—sites where virus is known to persist in some pastels for up to 25 months after inoculation.

b. Studies on immune responses and phagocytic activity of normal and AD affected mink. See NIAID Project 175.

c. The virus. Continued efforts to demonstrate AD virus in tissue culture were unsuccessful. For example, neither macroscopic nor microscopic cytopathic effects were observed in primary explants of virus-bearing tissues from infected mink or in a variety of normal mink cell monolayers inoculated with AD virus.

In other experiments, cultures of splenic macrophages and of suspensions of splenic cells from mink were maintained for up to 2 weeks. As measured by viable cell counts, proliferation of such cells appeared doubtful. Survival of cells from spleens of mink affected with AD did not differ from that of splenic cells from normal mink. Because the spleen and mesenteric lymph node are rich sources of virus in infected mink, cultures of cells from these organs are being used in further experiments directed toward virus isolation, fluorescent antibody reactions, and electron microscopic examination.

4. Progressive pneumonia.

a. The disease process. Thus far, little progress has been made in understanding the pathogenesis of progressive pneumonia. This is so despite our ability to induce the disease experimentally in some sheep and goats and to grow the causative virus in tissue culture. Too little is known about the conditions necessary for the consistent production of the disease experimentally to allow a definitive study at this time. Moreover, virus assay methods and procedures for evaluating the host's immune responses to the virus are still not adequate.

In an effort to test one variable, the host, we inoculated 2 groups of Cheviot lambs to determine their susceptibility. This breed, which is seldom found in areas where the natural disease is recognized, was chosen mainly because of its small size. One group inoculated intravenously with a 10% lung suspension known to cause the disease in other sheep has remained free of clinical signs of progressive pneumonia for 3 years. The other group inoculated by the same route with tissue culture virus likewise has remained clinically normal during the same period. These preliminary observations suggest that variation in breed susceptibility exists. To what extent this, and not regional differences in environment and flock management practices, accounts for the limited geographic distribution of the disease in the United States is not known.

In further attempts to determine the distribution of virus in tissues of naturally and experimentally infected sheep and goats, virus was isolated and grown in tissue culture repeatedly. Virus was isolated most often from lung homogenates, and these usually yielded the highest titers (10^3 to 10^6 $TCID_{50}$/ml). Less often, virus was isolated from homogenates of lymphocytic tissues, such as lymph nodes and tonsil. In these tissues, titers of virus were rarely greater than 10^3 $TCID_{50}$/ml. Virus was never isolated from central nervous tissue. This infrequent isolation of virus from tissues other than lung supports the view expressed previously that nonpulmonary tissues probably play a minor role in the pathogenesis of the disease.

b. Antibody responses. Recent attempts to demonstrate neutralizing and complement fixing antibodies in sera of experimentally infected sheep and goats failed to confirm results from earlier studies. At present, no explanation can be offered for this discrepancy. A more promising method for assessing the humoral immune response in this disease was developed by Dr. Kenneth Takemoto (LVD-NIAID) in collaboration with personnel here. This method involves the demonstration of serum anti-viral fluorescent antibodies. It will be useful in further studies on the experimentally infected sheep now available here.

c. The virus. The most sensitive tissue culture system for assaying virus continued to be a low-passage line of lamb testicular cells. When the assay was performed with an agar overlay, formation of polykaryocytes was found to be directly related to the virus dilution. The virus did not form plaques in this system. Optimum conditions for the assay system are still being investigated, as are other systems. A better system for assaying this virus is still needed.

Significance to biomedical research and the program of the Institute:

After decades of neglect, the discipline of comparative pathology underwent a kind of renaissance beginning about 1960. Since then, biomedical interest in naturally occurring diseases of animals has steadily increased. With much anticipation, concerned persons began to look to the day when information on animal diseases would contribute to an understanding of counterpart diseases of man. Although this revived interest in comparative pathology has indeed provided some valuable clues, the full potential of the comparative approach has yet to be realized. One small facet that already has contributed significantly to concepts about the mechanisms of human disease is exemplified by the group of animal diseases under study in this project—the so-called slow viral diseases. Their recognition during the past 10 years as unusual examples of host-virus relationships has had an appreciable impact on the mainstream of biomedical thought, especially as it concerns the cause and pathogenesis of chronic diseases of man.

Prevailing circumstances have forced biomedical investigators to address themselves to this important public health problem. In doing so, they are now seriously considering viruses as a probable cause of some chronic human diseases. Observations on the animal diseases, however fragmentary they may be, are largely responsible for this re-direction of effort. Therefore, studies such as this project, which is devoted mainly to characterizing animal diseases, are relevant to the central themes of modern biomedical research and should be sustained.

Proposed course of project:

Because much remains to be done, the animal work that forms the core of the project, however slow-moving and costly it may be, must certainly be maintained. The kind of information being sought can not perforce be obtained from purely in vitro studies or from experiments conducted solely in small laboratory animals.

Studies in progress will be continued. Some of the new studies contemplated for the coming year are:

1. Scrapie

a. An attempt will be made to determine the presence and tissue distribution of virus in lambs and young adult sheep from families of Suffolks known to have a high incidence of the disease.

b. The theory that scrapie virus includes a double-stranded nucleic acid moiety will be put to direct test in extraction experiments.

[illegible]. Encephalopathy of mink. Attempts will be made to adapt goat passage virus to mice.

3. Aleutian disease. The response of mink to nucleic acid antigens and the effect that sensitization to these antigens has on the course of the disease in both sapphires and pastels will be studied in a pilot experiment.

4. Progressive pneumonia. The anti-viral fluorescent antibody test developed by Dr. Takemoto will be used to assess the humoral immune response in experimentally infected sheep and goats. The nature of the immunoglobulins involved in this test will be investigated in cooperation with the Immunology Section.

Publications:

Takemoto, K. K., Mattern, C.F.T., Stone, L. B., Coe, J. E., and Lavelle, G.: Antigenic and morphological similarities of progressive pneumonia virus, a recently isolated "slow virus" of sheep, to visna and maedi viruses. J. Virol. 7: 301-308, 1971.

Serial No. NIAID-184
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biological properties of Bordetella pertussis antigens

Previous Serial Number: Same

Principal Investigators: J. J. Munoz and R. K. Bergman

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	4 2/12			4 2/12
Professional:	10/12			10/12
Other:	3 4/12			3 4/12

Project Description:

Objectives:

The main objectives have been: 1) to purify and identify biologically active substances from Bordetella pertussis, 2) to study the mechanism of action of the histamine sensitizing factor (HSF), and 3) to study the adjuvant action of B. pertussis extracts.

Methods employed:

Standard immunological, pharmacological, biochemical and bacteriological techniques and physicochemical methods of fractionating biological materials are used.

Major findings:

1. Further identification of reaginic type antibody from mice. it was previously reported that the reaginic type of antibody stimulated in mice by B. pertussis extracts could be obtained in a fraction eluted from DEAE cellulose columns with 0.05 M phosphate buffer at pH 7.9. The 2-hour

PCA antibody (7S gamma globulins) was obtained in a fraction eluted at [illegible] M. At [illegible] M only small amounts of the reaginic antibody mixed with the 2-hour antibodies were obtained.

Further work on purification has proven, as in the case of the human nonmyeloma γE, to be very difficult. Disc electrophoresis, either qualitative or quantitative type, has yielded purer fractions, but on careful testing these fractions still contain γ_1-γ_2 globulins. However, it has been possible to immunize guinea pigs, but not rabbits, with these fractions to obtain specific absorbed antisera that will neutralize the reaginic antibodies. This absorbed antiserum fails to react with all the known immunoglobulins of mice ($\gamma_1 G$, $\gamma_2 G$, γM, γA). Absorption of rabbit antisera with γ_1-γ_2 invariably reduced the titer to 0. The guinea pig sera were made specific by absorbing them with normal whole mouse serum. The slight reduction in neutralizing titer by this treatment indicates that the antigen (reaginic type of globulin) is in very small concentration in normal mouse blood. In fact, even our most purified preparations contain extremely small amounts, since its presence cannot be shown by radioimmunoelectrophoresis. However, it is a distinct immunoglobulin because it has distinct heavy chain specificity and light chains similar to IgG globulins. From its concentration in normal serum and other properties, it seems to belong to a class similar to γE of man.

2. Passive anaphylaxis with 2-hour and 72-hour PCA antibody. With standardized solutions of reaginic-type antibody (72-hour PCA antibody) and with 2-hour PCA antibody, passively induced systemic anaphylaxis was studied in normal and in HSF-treated CFW mice. One hundred PCA units of 2-hour PCA antibody (7S gamma) induced only anaphylactic symptoms in normal mice, while 4 of 10 HSF pre-treated mice died. One thousand PCA units of the same antibody induced fatal sensitization in 50% of the normal mice and 80% of the HSF-treated group. Unexpectedly, as much as 1000 PCA units of reaginic type antibody failed to sensitize mice to fatal anaphylaxis whether the mice were normal or HSF-treated. Anaphylactic symptoms were observed in both groups sensitized with 1000 PCA units and only in the HSF-treated group sensitized with 100 PCA units of reaginic type antibody.

3. Further attempts to purify HSF. In previous reports, it has been pointed out that HSF becomes rather insoluble when purification reaches a certain point. At the suggestion of Dr. John Gander, we tried sodium pyrophosphate buffer, with relatively good success, to dissolve semi-purified preparations of HSF. Solutions of HSF in 0.05 M Na pyrophosphate pH 8.2 were then passed through G-200 and G-75 Sephadex columns. To our surprise, the active material was included even in G-75 Sephadex. Four main peaks were obtained in G-75 and activity was observed in the first two peaks. Only a minor portion of the activity was recovered, perhaps because at pH 8.2 the activity is gradually lost. Disc electrophoresis of the material in the first peak gave only one band which did not penetrate the gel and a number of trace materials which were difficult

to see. The second Sephadex peak gave many bands. These observations indicate that HSF in these preparations occurs in different molecular entities and that active HSF can be found in molecules of relatively small molecular weight. Antisera prepared in guinea pigs with these preparations neutralized HSF activity.

Significance to biomedical research and the program of the Institute:

The many biological effects of B. pertussis extracts are of importance because they touch on fundamental mechanisms involved in antibody production, autoimmune disease, allergic reactions, and protective mechanisms of specific and nonspecific nature. To completely purify the main factor or factors involved is of obvious advantage and importance. The marked enhancement of reaginic antibody stimulation by B. pertussis extracts offers a possibility of channeling the immune response in the direction of specific immunoglobulins. If this could be achieved, one would have an effective means of studying mechanisms of antibody formation at the cellular level.

Proposed course of project:

Our efforts in this project have been markedly reduced due to reduction in personnel in our section. We will continue our efforts to identify the histamine sensitizing factor and if successful to study all of its various biological activities.

Honors and Awards:

Dr. J. J. Munoz (see also Project 175)

Asked to participate on a round table discussion on adjuvants at the 1st International Congress of Immunology to be held in Washington, August 1971.

Reviewer for the journal Infection and Immunity.

Publications:

Munoz, J., Smith, R. F., and Cole, R. L.: Separation of biological activities. Introductory remarks. Some physical properties of histamine-sensitizing factor. In Regamey, R. H., De Barbieri, A., Hennessen, W., Ikic, D., and Perkins, F. T. (Eds.): Symp. Series Immunobiol. Standard. International Symposium on Pertussis, Bilthoven, Netherlands, 1969. Basel/München/New York, S. Karger, 1970, vol. 13, pp. 211-213 and 265-270.

Publications (Cont'd):

Ross, R. F. and Munoz, J.: Antigens of Bordetella pertussis. V. Separation of agglutinogen 1 and mouse-protective antigen. Infect. Immun. 3: 243-248, 1971.

In press:

Munoz, J.: Protein toxins from Bordetella pertussis. In Microbial Toxins. Comprehensive Treatise, Academic Press.

Bergman, R. K. and Munoz, J.: Effects of epinephrine, norepinephrine and isoproterenol against histamine challenge in Bordetella pertussis-treated and beta adrenergic blocked mice. Life Sci.

Serial No. NIAID-185
1. Rocky Mountain Laboratory
2. Serological Technology and Epidemiology Sections
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Agents of the psittacosis-lymphogranuloma-trachoma group (*Chlamydia*)

Previous Serial Number: Same

Principal Investigators: R. N. Philip and R. K. Gerloff

Other Investigators: Elizabeth Casper and L. A. Thomas

Cooperating Units: Dr. F. B. Gordon, Naval Medical Research Institute, Bethesda, Maryland
Dr. D. G. Waldhalm, University of Idaho Veterinary Research Laboratory, Caldwell
Dr. S. P. Wang, School of Public Health and Community Medicine, University of Washington, Seattle
Prof. B. R. Jones, Institute of Ophthalmology, University of London, England
Dr. F. W. Frank, College of Agriculture, University of Idaho, Moscow

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	5 6/12			5 6/12
Professional:	2 4/12			2 4/12
Other:	3 2/12			3 2/12

Project Description:

Objectives:

The general purpose of this project is to investigate relationships among the chlamydiae, to clarify the epidemiology of chlamydial diseases and determine their total importance to human health, and to apply technological advances to the laboratory study of these organisms.

More specific objectives include: 1) improved methods for isolating chlamydiae from clinical specimens, 2) isolation and characterization of new strains, 3) more luxuriant growth in embryonated eggs and cell cultures, 4) simpler methods for purifying and quantitating elementary body suspensions, 5) more sensitive and specific diagnostic reagents and techniques, 6) antibody surveys to determine prevalence of chlamydial infection in specific human and animal populations, 7) study of chlamydial interrelationships and characteristics of possible taxonomic value, and 8) study in laboratory systems of the immunogenicity of whole organisms and fractions thereof.

Methods employed:

A wide variety of techniques were employed, most of which are standard for chlamydial, rickettsial, and viral research. These include cultivation of chlamydiae in yolk sacs and various cell cultures; complement fixation, radioisotope precipitation, neutralization, and toxicity prevention tests; and observation of inclusions and elementary bodies in preparations stained by the Gimenez procedure or with fluorescein-conjugated antiserums.

Two relatively new procedures utilized were: 1) the micro-immunofluorescence (micro-IF) procedure of Dr. S. P. Wang for study of antigenic relationships, and 2) counting of elementary bodies by use of standardized latex particle suspension and Congo red negative stain. Antigenic typing with the micro-IF test gives results which parallel those from the more laborious mouse toxicity prevention test and immunization of monkeys against ocular challenge.

Major findings:

1. Antigenic interrelationships among LGV and TRIC chlamydiae. With the micro-IF test, chlamydial isolates from the Crow-Cheyenne Indian Reservation and from the District of Columbia (DC) have been typed.

Six serotypes (designated A through F) are now recognized among TRIC organisms. Our Crow strains are clearly type Ba and correspond to eye strains isolated from Indians in the Southwest by Hanna and associates. On the other hand, our DC TRIC isolates, while similar to one another, are antigenically quite distinct from previously classified strains and will probably comprise a new TRIC serotype along with several other heretofore untypable strains.

Two serotypes (designated I and II) are now recognized among LGV strains and these strains are antigenically related to the B-D-E complex of TRIC serotypes. All of our DC LGV strains tested thus far appear to be type II but are serologically distinguishable from previously classified LGV strains.

2. Immunologic studies in mice. The results of our cross-neutralization tests of LGV and neuropathogenic TRIC strains in mice (see previous annual reports) were reported in Boston at the 1970 Conference on Trachoma and Allied Diseases. The patterns of neutralization corresponded to findings obtained by others in serotyping by immunofluorescence, and provided additional support for categorizing LGV strains into at least 2 antigenic groups. By both neutralization and micro-IF, all of the fast-killing (f) TRIC strains were typed as LGV II. This has stimulated considerable controversy as to whether the f TRIC strains were derived from mixtures of LGV and TRIC particles or whether TRIC characteristics were lost as a result of laboratory manipulation.

The mechanism of serum neutralization of LGV chlamydiae is being studied in mice. That neutralization is a true antigen-antibody reaction is indicated by the type-specificity obtained and by the direct correlation of neutralization with other serologic tests. However, much neutralizing activity was lost when hyperimmune chicken serums were inactivated at 56°C for 30 minutes. Addition of fresh normal chicken serum or guinea pig complement fully restored neutralizing activity. It has long been recognized that neutralization of chlamydiae is, at best, of low order. This is partially explained by the fact that chlamydiae form infectious complexes with antibody. Addition of anti-species globulin to our neutralization system in mice markedly enhanced the degree of neutralization obtained with hyperimmune chicken serums.

3. Ovine abortion. Collaboration has continued with veterinary personnel of the University of Idaho in studies of chlamydia-caused abortion in sheep. All serologic testing, involving over the past year complement fixation tests on about 1300 serums from approximately 200 sheep, has been done at RML.

The effects of large doses of the ovine abortion agent given orally (PO) or intramuscularly (IM) and either 30 days before breeding or in the 3rd month of gestation were compared. The route of administration had less effect on percentage of abortions than did time of administration. The highest abortion incidence (58%) occurred in sheep exposed PO late in gestation. The time of exposure or route did not significantly affect the number of barren ewes resulting. Antibody response in ewes inoculated PO was greater than that in the IM group. In those animals inoculated late in gestation, titers peaked at the time when abortions were most frequent.

4. Chlamydial particle counting. A relatively simple and convenient method for counting elementary bodies has been developed. Latex particle suspension of known concentration is used as a reference standard. Chlamydial particle concentration is calculated from the chlamydial:latex particle ratio in a mixture containing specific volumes of the two kinds

of suspension, and the concentration of latex particles in the standard. Particles are visualized by ordinary light microscopy of smears negatively stained with Congo red. The procedure does not require complex laboratory equipment and the negative stain virtually eliminates erroneous readings due to loss of particles during staining. Since the chlamydial particles are not differentially colored from foreign bodies, satisfactory counting does require fairly clean preparations.

With the counting procedure it was determined, for example, that 1 chick embryo LD_{50} of meningopneumonitis agent comprises 12 particles.

5. Toxic effects of various chlamydiae on cell monolayers. This study has been continued, mainly for the purpose of repeating and expanding preliminary experiments to check thoroughly the validity of results.

The toxic phenomenon has been confirmed with all of 13 chlamydial strains (representing both species of Chlamydia) studied in 1 cell line and in all of 4 cell lines tested with 2 chlamydial strains. Cell monolayers inoculated with concentrated chlamydial particle suspensions and centrifuged at 1700 X G for 1 hour were destroyed in 16 hrs or less. Because the destruction occurred long before inclusions matured in the normal developmental cycle, it has been termed a toxic effect.

Toxicity of an agent for cells was not dependent on its ability to produce inclusions in those cells. It did, however, appear to be highly dependent on the presence of infectious particles. The toxic agent was relatively thermolabile and could be neutralized by specific antiserum. Massive doses of penicillin and sulfadiazine were generally ineffective against it, even in cell-agent systems where minute amounts interfered severely with development of inclusions. Comparison of the susceptibility of cell monolayers and intravenously inoculated mice indicated that monolayers provide a more sensitive tool for titration of chlamydial toxicity but are less suitable for testing suspensions containing high proportions of host tissue. Contaminating tissue obscures the cells in monolayers.

If a specific factor in chlamydial particles is responsible for the toxic effect, this factor lacks properties of either an exotoxin or bacterial endotoxin. It resembles more closely the toxic substance associated with rickettsiae.

Significance to bio-medical research and the program of the Institute:

A practicable means of serotyping, such as the Wang micro-IF test, is contributing to our understanding of chlamydial infections of man. "Senior" and "junior" antigenic relationships (similar to the situation with type A influenza viruses) are being identified which permit hypotheses as to the evolution of strains and the relationship of ocular to genital infection. For example, antiserums prepared against our Crow trachoma strains (type Ba) are broadly reactive against TRIC type E and LGV II strains, which are genital organisms. The reverse is not true. This suggests that the type Ba ocular strains may have recent origins from the genital tract of man. Certain facets in the epidemiology of trachoma among American Indians support such hypothesis. The disease is mild. The few outbreaks that occur are self-limiting, as if the infecting organism is poorly suited for perpetuation by eye-to-eye transfer. Perhaps the genital tract is an important reservoir of infection among Indians.

Interest in the toxicity of chlamydial suspensions for cell cultures arose because of its practical importance in cultivation of these organisms in such cultures. When inocula were too great, yields at harvest were low because of early, severe cytopathogenic effects on the host cells. This importance still exists. Another area of importance has come into view; namely the usefulness of a toxicity neutralization test in cell cultures for taxonomic studies. Such tests, previously performed in mice, can be done more conveniently and economically in cell cultures.

The Congo red-latex method for counting particles is especially suitable for cell culture-produced chlamydial suspensions because they initially are relatively clean and require little additional treatment. Hence the method will be useful, for example, in standardization of RIP antigens in terms of radioactivity per particle.

Proposed course of project:

Serotyping with the micro-IF test will be continued along the lines described. In addition, typing of patient serums will be done in an attempt to determine whether the antigenic nature of the infecting strain of chlamydia can be identified in this manner. Mammalian strains of Chlamydia psittaci will also be examined by this procedure to determine whether specificity exists. If so, this test may prove to be a useful means for clarifying the antigenic relationships among chlamydiae infecting domestic animals, particularly cattle and sheep.

Whether addition of antiglobulin facilitates detection of neutralizing antibody in patient serums titrated in mice, as it does in the case of hyperimmune chicken serums, will be further explored.

The role, if any, that possible mycoplasma contamination might play in the chlamydial toxicity phenomenon in cell cultures will be investigated. Also the limits of specificity of the toxicity neutralization test will be checked, particularly as it applies to differentiation of certain TRIC and LGV strains. RIP tests will be continued with various purposes in mind, firstly to examine another group of serums soon to be received from patients with Reiter's syndrome; secondly, to learn whether more reactive antigens can be prepared from chlamydiae other than the meningopneumonitis agent and whether higher RIP antibody titers can be obtained by using antigen prepared from a strain more closely related to that which stimulated production of the antibody being assayed.

Publications:

Meinershagen, W. A., Waldhalm, D. G., Frank, F. W., Thomas, L. A., and Philip, R. N.: Efficacy of combined bacterins for experimental immunization of sheep against ovine vibriosis and chlamydial abortion of ewes. *Amer. J. Vet. Res.* 32: 51-57, 1971.

In press:

Gerloff, R. K., Ritter, D. B., and Watson, R. O.: Chlamydial particle counts by negative staining with Congo red. *J. Infect. Dis.*

Philip, R. N. and Whitlock, J. J.: Immunologic studies of lymphogranuloma venereum and mouse-adapted TRIC chlamydiae in mice. *Excerpta Medica*

Waldhalm, D. G., Frank, F. W., Meinershagen, W. A., Philip, R. N., and Thomas, L. A.: Lambing performance of ewes inoculated with *Chlamydia* sp. before and after breeding. *Amer. J. Vet. Res.*

Serial No. NIAID-186
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The biology of microbial agents in arthropods

Previous Serial Number: Same

Principal Investigator: W. Burgdorfer

Other Investigators: L. P. Brinton, R. A. Ormsbee, M. G. Peacock, and L. A. Thomas

Cooperating Units: NAMRU-3, Cairo, Egypt
Mr. J. D. Gregson, Canada Department of Agriculture, Kamloops, B.C.
Major M. G. Groves, Walter Reed Army Institute of Research, Division of Veterinary Medicine, Wash., D.C.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 8/12			3 8/12
Professional:	1 6/12			1 6/12
Other:	2 2/12			2 2/12

Project Description:

Objectives:

To study dynamics of viral, rickettsial and bacterial infections in arthropod vectors. Studies of morphology, histology and fine structure of arthropod tissues are coordinated with research of interactions between such tissues and microorganisms. Specifically investigated are 1) development of pathogens within growth-supporting cells, 2) cellular response to infection, 3) effects on physiological characteristics of microorganisms resulting from pathogen-arthropod association, and 4) factors supporting or inhibiting development and survival of infectious agents.

Research during the past year included 1) various aspects of ovarian maturation in Dermacentor andersoni, 2) the fine structure of normal hemocytes of ticks, 3) the role of tick hemocytes in development of Colorado tick fever (CTF) virus and of various rickettsial agents (Rickettsia rickettsi, R. montana, R. prowazeki), 4) attempts to infect ixodid ticks with R. prowazeki, 5) vector relationships of R. canada, 6) development of R. conori in Hyalomma dromedarii and Amblyomma cohaerens, 7) development of Ehrlichia canis in Rhipicephalus sanguineus, and 8) continued attempts to identify and isolate rickettsialike organisms in ticks from British Columbia, Canada.

Methods employed:

In addition to routine techniques of medical entomology, bacteriology and virology, fluorescent antibody (FA) procedures are applied to studies of viral, bacterial and rickettsial infections in arthropods. Electron microscopy is used to characterize subcellular structures of normal and infected arthropod tissues and to determine morphology of microorganisms during intracellular development.

Major findings:

1. Gross histologic and electron microscopic aspects of ovarian maturation in D. andersoni were established for unfed and engorged nymphs and for unfed, newly molted, feeding and engorged adults through completion of oviposition. Interstitial cells, whose processes surround oogonia in unfed nymphs, become segregated in the adult as an epithelium that lines the ovarial lumen. Subsequently, funicular cells differentiate from this epithelium. Oocytes undergo most rapid development while attached as extensions to funicular cells and exposed to hemocelic fluids during and after completion of engorgement. Upon complete development, oocytes pass via funical cells into the lumen of the ovary. Later, they gain access to paired oviducts and eventually to the utero-vaginal complex from where they are oviposited.

Observations on developmental morphology and fine structure of normal oogonia and oocytes revealed an overlap of intrinsic and extrinsic phases in oocyte development. The intrinsic or intra-oocytic phase, already evident in unfed adult females, is characterized by heavy emission of ribonucleoprotein from the nucleus, coalescing of ribosomes with vesicles budded as saccules from dictyosomal cisternae to form dense vesiculate bodies, and by morphogenesis of mitochondria to form membrane-limited multivesiculate bodies. Extrinsic or extra-oocytic factors consist of micropinocytosis of hemocelic fluids following formation of a microvillar brush border around oocytes, 4 to 6 days after attachment. Ingested fluids pass via micropinocytotic tubes to membrane-limited amorphous reservoirs in the cortical ooplasm. Subsequent fusion of these bodies with dense bodies of intrinsic origin forms proteid-yolk granules. Intercellular bridges were observed between oogonia and oocytes of nymphal and adult D. andersoni ovaries. Although the exact physiologic function of these formations is unknown, it is speculated that they contribute to the synchronous development of germ cells.

2. Based on morphologic appearance and fine structure of hemocytes in D. andersoni, the following cell types were established: prohemocytes, spherule hemocytes, and plasmatocytes. Prohemocytes were smallest in size and always possessed a high nucleo-cytoplasmic ratio and rough endoplasmic reticulum. Spherule hemocytes were most numerous and were divided into 4 types (sensu lato), on the basis of their gross morphology, cytoplasmic fine structure and inclusions. Each type contained prominent dictyosomes with numerous associated vesicles. Fusion of such vesicles and contribution of material from cisternae of the rough endoplasmic reticulum resulted in formation of inclusion bodies. Plasmatocytes were always of irregular form and usually possessed filopodia (pseudopodia). Only this cell type had a propensity for phagocytosis and apparently serves a scavenging role in the hemocele.

3. Studies have been initiated but are still of preliminary nature to determine the type of hemocyte that exhibits greatest propensity for acceptance and transport of viral (CTF) and rickettsial agents (R. rickettsi, R. prowazeki and R. montana). Emphasis is placed on the cellular mechanisms involved in releasing these pathogens.

4. Partial success was obtained in our attempts to infect ixodid ticks with the Cairo-3 strain of R. prowazeki. After feeding on rickettsemic Microtus and/or guinea pigs, larval Hyalomma dromedarii, Rhipicephalus simus, R. evertsi, Amblyomma variegatum and D. andersoni ingested sufficient rickettsiae to be detected in the digestive system, either through injection of tissue suspensions into test animals or microscopically by FA techniques. However, this infection was mild and of temporary nature only; in no instance did it become generalized or persist through the adult stage.

5. Extremely high mortality from massive multiplication of R. canada in transovarially infected larval D. andersoni has previously been recorded. It has now been established that this agent also affects the biological processes of D. variabilis; infected female ticks of the F_1 generation invariably produced small batches of eggs that failed to develop. Electron

microscopic studies revealed considerable disruption of intracellular organization of several tissues. This breakdown of hostcell cytoplasm is manifested initially by the typical "halo" around each rickettsia, and later by a general deterioration of the cytoplasm.

The lone-star tick, Amblyomma americanum, also appears a potential vector of R. canada. Larval ticks that fed on rickettsemic Microtus became infected and maintained infection transstadially as well as transovarially. Minimal dosage requirement for infection of this species of tick appears much greater than those for D. andersoni and D. variabilis.

6. Several lines of naturally infected D. variabilis (TVA-LBL origin) and Amblyomma cohaerens (Ethiopia origin) and one line of Hyalomma dromedarii (Egypt) were established to study behavior and development of their respective rickettsial agents. Data available so far indicate that, in contrast to reports in the literature, D. variabilis transmits R. rickettsi to 100% of its progeny.

7. Studies concerning identification and development of Ehrlichia canis in Rhipicephalus sanguineus are in progress. Attempts to infect nymphal ticks by feeding on infected dogs have been successful as indicated by infectiousness of ticks in the adult stage. However, Haemobartonella canis infection in dogs used to maintain tick colonies (at Walter Reed Research Institute) renders uncertain current microscopic findings. Experiments with "clean" dogs, and with ticks that have been maintained on rabbits and guinea pigs only, have been initiated.

8. Additional surveys of D. andersoni from British Columbia, Canada, revealed that a relatively large percentage from that area contain at least 2 different rickettsialike agents that are nonpathogenic for the usual test animals and therefore cannot be isolated. One agent is more bacillary and occurs in all tissues, particularly in Malpighian tubules and ovary. The other, highly pleomorphic and more rickettsialike, chiefly infects ovarian tissues and epithelial cells of the midgut. Both organisms appear antigenically related to spotted fever group rickettsiae by FA cross-staining. However, efforts to prepare conjugates by injecting infected tick material into guinea pigs and rabbits were unsuccessful. Electron microscopy revealed fine structures similar to those of R. rickettsi rather than Wolbachia.

Significance to bio-medical research and the program of the Institute:

Detailed knowledge of developmental morphology, histology and fine structure of arthropod tissue is essential for meaningful interpretation of potential tissue-parasite interactions. The ovarian tissue, in particular, supports growth of bacterial and rickettsial agents to such an extent that these organisms are passed transovarially to the progeny of infected ticks. Intercellular bridges undoubtedly serve as channels by which such pathogens are disseminated throughout the ovary.

Hemocytes are the most important tissue cells for the development of pathogens in ticks, for they are responsible for disseminating viral, bacterial and rickettsial agents upon penetration of the gut wall. Although the physiologic functions of the various cell types are still unknown, plasmatocytes, in particular, appear to be phagocytic. It remains to be investigated whether hemocytes in general represent a defensive mechanism against microbial agents.

Our assumption (Annual Report 1969/70) that infection of ticks with *R. prowazeki* depends on the concentration of rickettsiae ingested proved correct. The present findings with the highly virulent Cairo-3 strain confirm, to some extent, Dr. Reiss-Gutfreund's experimental data. However, in view of the fact that domestic animals do not experience a detectable rickettsemia, these data still do not support the hypothesis that ticks and domestic animals are involved in an extrahuman cycle of epidemic typhus.

Presumptive evidence that *R. canada* produces in man a disease similar to spotted fever has recently been established. Ticks, possibly *D. variabilis* and *A. americanum*, have been suggested possible vectors. Our experimental data indicate that both species are potential vectors. However, adverse effects on tick development as result of massive rickettsial growth suggest that association of *R. canada* with these species of ticks (also *D. andersoni*) is not a natural one.

Tropical canine pancytopenia among military dogs in Vietnam has rekindled interest in the relationship between its causative agent, *Ehrlichia canis*, and the tick vector, *Rhipicephalus sanguineus*. Since little is known on the nature and biology of this rickettsial agent, particularly its development in ticks, we appreciate the opportunity of doing cooperative research with investigators of the Division of Veterinary Medicine at Walter Reed Army Institute of Research.

Presence of nonpathogenic rickettsialike microorganisms in a large percentage of *D. andersoni* from British Columbia, Canada, may have a direct relationship to the rare occurrence of *R. rickettsi* in ticks of that area. An interference phenomenon, similar to that reported last year between these nonpathogenic microorganisms and the spotted fever agent may be responsible for inhibiting growth and dissemination of the latter.

Proposed course of project:

Electron microscopic investigations of normal and infected tick tissue systems will be continued with special emphasis on hemocytes infected with CTF virus and various rickettsial agents. In addition, the following projects will be initiated: 1) development and subcellular relationship of CTF virus in *D. andersoni*, and 2) a study on interactions between rickettsial antibodies and antigens in leucocytes, lymph and spleen tissues of *R. rickettsi*-infected *Microtus* or *Peromyscus*.

Although PL-480 Project No. 03-008-1 will be terminated June 10, 1971, studies related to experimental infection of various species of ixodid ticks with *R. prowazeki* will be continued until observations and results with nymphal ticks become available.

Considering the possibility that arthropods other than ticks may be involved in the ecology of *R. canada*, behavioral studies will be extended to the rat flea, *Xenopsylla cheopis*. Preparations for establishing a productive flea colony are presently under way.

Since practically nothing is known on the morphological appearance of *Ehrlichia canis* in tissues of infected *Rhipicephalus sanguineus*, our initial studies will be limited to differential examination of tissues from infected and normal ticks. Once the agent is recognized, its development can be studied and additional projects can be initiated to establish its biological characteristics and to analyze its ill-defined relationship to rickettsiae.

Attempts will be made to isolate rickettsialike microorganisms in *D. andersoni* from British Columbia by culturing infected tissues on primary tick tissue cultures and chick-embryo fibroblasts.

Honors and Awards:

Dr. Willy Burgdorfer

Continued to serve as an Associate Member of the Commission on Rickettsial Diseases, Armed Forces Epidemiological Board.

Invited to present paper "Ticks as Vectors of *Rickettsia prowazeki* (Current status of experimental research)" at Annual Meeting of the Commission on Rickettsial Diseases, AFEB, Washington, November 12-13, 1970.

Succeeded Dr. C. B. Philip as Project Officer of PL-480 Project No. 03-008-1.

By request reviewed research manuscripts for Journal of Epidemiology and Journal of Medical Entomology.

Continued to serve as Faculty Affiliate for the Department of Zoology, University of Montana, Missoula.

Dr. L. P. Brinton

Elected member of the Electron Microscopy Society of America.

Publications:

Burgdorfer, W.: Worldwide research on human and animal diseases caused by tickborne rickettsiae. Misc. Publ. Entom. Soc. Amer. 6: 339-344, 1970.

Burgdorfer, W. and Brinton, L. P.: Intranuclear growth of *Rickettsia canada*, a member of the typhus group. Infect. Immun. 2: 112-114, 1970.

Burgdorfer, W.: Hemolymph test. A technique for detection of rickettsiae in ticks. Amer. J. Trop. Med. 19: 1010-1014, 1970.

Brinton, L. P. and Burgdorfer, W.: Fine structure of *Rickettsia canada* in tissues of *Dermacentor andersoni* Stiles. J. Bact. 105: 1149-1159, 1971.

In press:

Brinton, L. P. and Oliver, J. H., Jr.: Ovarian maturation in *Dermacentor andersoni* Stiles (Acari: Ixodidae). I. Gross anatomical histological and cytological aspects of development. J. Parasit.

Brinton, L. P. and Oliver, J. H., Jr.: Ovarian maturation in *Dermacentor andersoni* Stiles (Acari: Ixodidae). II. Fine structure of oogonial and oocyte maturation, including vitellogenesis and vitelline membrane formation. J. Parasit.

Serial No. NIAID-187
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Host-ectoparasite relationships

Previous Serial Number: Same

Principal Investigator: C. M. Clifford

Other Investigators: J. F. Bell, B. M. Hestekin, J. E. Keirans, and C. E. Yunker

Cooperating Units: Department of Zoology, University of Montana, Missoula: Dr. L. G. Browman and Mr. M. E. Shaw
Dr. W. A. Nelson, Canada Department of Agriculture Research Station, Lethbridge, Alberta

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	1 8/12			1 8/12
Professional:	9/12			9/12
Other:	11/12			11/12

Project Description:

Objectives:

Studies are directed toward classification of certain phases of host/arthropod relationships. Emphasis is centered on identifying the mechanisms by which animals become resistant to feeding of arthropods and factors governing responses of parasitized animals to infection with various microorganisms.

Methods employed:

The host-parasite system developed at the RML whereby measurable and predictable populations of lice can be developed on various strains of mice by restriction of self-grooming is extensively used. At times other laboratory animals are utilized and a variety of microbiologic, immunologic and entomologic techniques are employed.

Major findings:

1. Work at the RML during the past year has centered around the development of more precise methods for studying the effects of lice on mice.

Weight losses of RML strain of mice were not significant if primary seedings were small (20-30 seeded/mouse) in a 14-week period of observation. Louse infestations were higher in males than in females and the percent of packed cells decreased more in the former than the latter.

In louse-infested C_{57} BL mice observed during a 9-week period weight variations were not significant; however, the percent of packed cells decreased progressively through the 5th week, and 95% of the mice died during the 4th and 5th weeks.

Another series of experiments was conducted to determine the effect of known numbers of lice on mice. Approximate weight/number ratios of lice were determined and the average weight of a louse was 29.4 µg. Three-week-old RML mice weighing 10.5 gms were seeded with 80 mg of lice per mouse and 85% died by day 13. In 4-week-old mice weighing 16.0 gms seeded with the same number of lice 70% had died by day 13. In 5-week-old mice weighing 24.5 gms which received the same burden only 15% had died. Next the amount of lice put on each mouse was varied with the following results: 14-15 gm mice seeded with 240 mg of lice/mouse all died by day 3; mice of similar weight that received 190 mg of lice/mouse were all dead by day 4, and 140 mg of lice/mouse caused 40% of the mice to die by day 4; the rest survived until day 13 and 14 when the remaining 60% succumbed.

2. Some further attempts to detect antibody response to louse infestation were undertaken. Lice were pressurized at 15,000 Psi at 5-10°C following emulsification in an Omni-Mixer and the resultant suspension was prepared in saline. Rabbits were immunized with this material by either IV injection, subcutaneous injection in complete Freund's, or both. The subcutaneous injections were the most satisfactory for producing antibodies. Examination of these serums by immunodiffusion (Ouchterlony technique) revealed 4 and 5 precipitin bands. However, no degree of immunity was shown by passive transfer of serums having these bands or by actively immunizing mice with whole louse suspension.

Protection of mice against sudden death from overwhelming louse burdens by transfer of serum or lymph node, spleen or macrophage cells from immune mice has been attempted but without success to this point.

Significance to bio-medical research and the program of the Institute:

The aim of this project is an understanding of the mechanism whereby mice become resistant to the feeding of lice. This study has been possible because of the mouse-louse experimental system developed at the RML. Information from the study could aid in development of methods for protecting man and animals against deleterious effects of the feeding of arthropods. The mouse-louse system offers a unique opportunity to study the development of resistance to arthropod feeding since the experiments can be conducted at a relatively low cost.

Proposed course of project:

Dr. Nelson will continue to study the histopathology of mice that are infested with lice. He will devote more time to studying these effects in strains of mice that exhibit varying degrees of susceptibility to these parasites.

Mr. Michael Shaw should complete his studies on the genetic transfer of the capacity of acquired resistance of mice to lice during the next year.

Work at the RML will focus on attempts to demonstrate antibody to lice in mice that have been infested. It is hoped that the use of known weights (or numbers) of lice, which will give predetermined death rates, will facilitate these studies.

Publications:

Lodmell, D. L., Bell, J. F., Clifford, C. M., Moore, G. J., and Raymond, G.: Effect of limb disability on lousiness of mice. V. Hierarchy disturbance on mutual grooming and reproductive capacities. *Exp. Parasit.* 27: 184-192, 1970.

Bell, J. F., Moore, G. J., Clifford, C. M., and Raymond, G. H.: Dry gangrene of the ear in white mice. *Lab. Anim.* 4: 245-254, 1970.

Serial No. NIAID-188
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Vector-pathogen relationships

Previous Serial Number: Same

Principal Investigator: C. E. Yunker

Other Investigators: C. M. Clifford, L. E. Hughes, J. E. Keirans, R. A. Ormsbee, C. R. Owen, and L. A. Thomas

Cooperating Units: Dr. S. Baron, LVD, NIAID, NIH
Dr. M. N. Kaiser, NAMRU-3, Cairo, Egypt

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 1/12			3 1/12
Professional:	1 6/12			1 6/12
Other:	1 7/12			1 7/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 Agreement 03-016-1	C. E. Yunker	NAMRU-3 Cairo, Egypt	M. N. Kaiser	$19,647

Project Description:

Objectives:

1) To investigate the host-range, cellular relationships and growth dynamics in arthropods or their cells of pathogenic agents transmissible by vectors among man or animals, 2) to develop systems of vector- or vector-related-tissue and cell culture and to apply these systems to the study of arthropod-borne disease and vector-pathogen relationships, 3) to define the microenvironment to which pathogenic microorganisms are subjected when ingested by tick vectors, 4) to provide basic biochemical information necessary for the progress of developmental tick tissue culture studies.

Methods employed:

Disease agents of primary interest are arthropod-borne viruses and, to a lesser extent, rickettsias, which are transmitted by Acarina and Diptera. Thus, conventional basic techniques in laboratory arbovirology and experimental acarology and entomology are employed. A major effort involves development and use of arthropod tissue and cell cultures. Standard and specially devised biochemical and physiological techniques are used to determine the nature of tick body fluids. Resultant findings are employed in the preparation of tick tissue culture medium in efforts to improve the growth supporting characteristics of the medium.

Major findings:

1. Since last report we acquired a newly established line of cells from the mosquito, Culex quinquefasciatus, and, in addition, have developed a rapid method for preparing primary cultures of tick hemocytes (see below). Eighteen viruses have been tested for their ability to propagate in these systems or in others currently in use. The C. quinquefasciatus cell line was shown to support growth of the mosquito-borne viruses, St. Louis encephalitis (SLE), Sindbis and Dengue 2, but not of the tick-borne viruses, Langat, Powassan, Tuleniy, Lone Star, Silverwater or Colorado tick fever (CTF). Thus, the pattern of selectivity previously determined for other mosquito-cell lines (Aedes albopictus and Ae. aegypti) seems to hold true for this line; i.e., culicine-borne, but not tick-borne, viruses are capable of growth in mosquito cells in vitro.

Primary cultures of tick hemocytes proved more limited in their ability to support growth of viruses than tissue cultures of whole tick viscera. CTF, Kemerovo, Powassan and Langat viruses, but not Lone Star, Silverwater or Tuleniy viruses grew in hemocyte cultures. All of these viruses propagate in primary cultures of tick viscera. Two mosquito-borne viruses, SLE and Eastern equine encephalitis, also failed to grow in tick-hemocyte cultures.

2. Demonstration of Kemerovo-Chenuda group agents in ticks in the United States (see NIAID-181) has prompted us to investigate the affinity of viruses of this group for arthropod cells *in vitro*. To date we have tested Kemerovo, Chenuda, Wad Medani and Sixgun City viruses for growth in 4 insect cell lines. Although results are thus far negative, loss of virus titer of some of these agents after frozen storage makes these results equivocal. Further tests are planned.

3. The search for agents capable of producing cytopathogenic effects (CPE) on insect cells has continued. Preliminary evidence of CPE was seen in *C. quinquefasciatus* cells after infection with Dengue 2 and Sindbis viruses. These viruses are known to cause CPE in *Aedes* cells.

4. A search for interferon or an interference-type phenomenon in mosquito cells was continued. This year we were fortunate to obtain the collaboration of Dr. S. Baron, LVD, NIAID. *Aedes albopictus*, *Ae. aegypti* and *C. quinquefasciatus* cells were exposed to the interferon inducing agents, Chikungunya and West Nile (WN) viruses and *Coxiella burneti*, and to 2 synthetic polynucleotides, Poly I·C and Poly A·U. Cultures were then challenged with 3 arboviruses: vesicular stomatitis (Indiana strain), CTF, and Sindbis. Slight, but consistent, reduction of challenge virus titers was seen in 3 of these systems. A repeat of this experiment is in progress.

5. We previously showed that insect cell cultures were satisfactory to excellent substrates for the growth of certain pathogenic rickettsiae, namely *C. burneti*, *Rickettsia prowazeki*, and *R. rickettsi*. This year *R. tsutsugamushi* (Karp strain) was found to grow extremely well in 3 mosquito cell lines: *Ae. aegypti*, *Ae. albopictus*, and *C. quinquefasciatus*. In all 3 systems, numbers of infected cells and numbers of organisms within them increased, tending to be highest between the 5th and 8th weeks. Low to moderate CPE, evidenced by degenerative changes in cells, accumulation of cellular debris and reduction of cell numbers, were seen. Assay of rickettsial growth in adult mice revealed an increase of 3 1/2-4 1/2 dex beyond the amounts inoculated. Some evidence of prolonged survival but not growth of the organism in *Antheraea eucalypti* cells was seen.

6. Significant effort was devoted to the emergent problem of monitoring the purity and identity of arthropod cell cultures. Chromosome number and morphology have proven valuable in distinguishing mosquito and moth cells from all others, arthropod and mammalian, under cultivation in our laboratory. Other methods must be sought for specific identification.

7. A colony of *C. tarsalis*, an important arbovirus vector in the western United States, was established and efforts were initiated to develop satisfactory culture methods for tissues and cells of this species. Eighty-eight primary cultures from young adult gonads of this species were

prepared and maintained for as long as 8 1/2 months in a medium containing lactalbumin hydrolysate. Attempts to subcultivate cells from these cultures failed. Renewed efforts to establish a line of cells from C. tarsalis larvae will be made if these larvae can successfully be reared axenically. To date this has not been reported for this species of mosquito.

8. The substrain of Ae. albopictus cells adapted to medium free of serum (see last report) is presently in its 30th passage. Its growth rate in stationary cultures is somewhat slower than the serum-dependent parent strain and unlike the latter fails to grow satisfactorily in spinner cultures. Its reaction to infection with viruses and rickettsias, however, is not obviously different than the parent line.

9. A rapid and simple technique for preparing and maintaining primary cultures of tick cells (hemocytes) has been devised. Cells may be maintained for 72-74 days in viable condition as evidenced by their ability to support growth of CTF virus. Previously, hemocytes have rarely survived more than one week in vitro.

10. Progress of PL-480 Agreement No. 03-016-1 included:

a. Completion of a definitive study of the biology and histological anatomy of Argas arboreus. Defined were the quantitative aspects of digestion and excretion at various ambient conditions, relationship of feeding to number of life stages, sex ratios and oviposition rate, and the anatomy and microstructure of the feeding apparatus and alimentary tract of the tick.

b. Completion of a study detailing the major inorganic composition of cell-free body fluids of 4 species of ticks representing the 2 major families. It was found that hemolymph ionic composition is held proportionately constant in the various developmental stages of all 4 species. Hemolymph sodium and chloride levels are higher than those in ingested food, but levels of potassium, iron and zinc are lower. Differences in ionic levels between various body fluids and those of the ingested bloodmeal are due to concentration and dilution processes controlled by the ionic regulatory system of each species. The coxal glands of Argas spp. play an important role in these processes by the elimination of excess water and electrolytes through coxal fluid secretions. It is indicated that ticks have the ability to tolerate relatively large changes in ionic composition.

c. Physiological studies on water balance metabolism of 2 Argas spp. revealed the important role of the integument, particularly the epicuticular wax layer, in restricting water-loss. Spiracular water-loss is under investigation, and experiments are being undertaken to determine critical equilibrium humidities and water-gain from the atmosphere of all stages of these ticks.

d. Biochemical studies of cell-free fluids of 2 species of ixodid and 2 species of argasid ticks were continued. Liquid-gas chromatography was shown to be more sensitive than thin layer chromatography for quantitative and qualitative analysis of amino acids of tick fluids. By the former method 12 amino acids were detected in the hemolymph of A. persicus, whereas only 8 were found by the latter one. These 12, also present in eggs of A. arboreus, exceed the number commonly detected in most insects. Quantitative differences in total lipids, phospholipids and sterols between host-blood gut contents and hemolymph (also of coxal fluid for the 2 Argas spp.) were determined, and a comparative study of glucose content of body fluids was completed. A biochemical study of tick excreta has been started and recently begun radioisotopic studies on incorporation of tyrosine and acetate in tick proteins and lipids, respectively, are yielding preliminary data.

Significance to bio-medical research and the program of the Institute:

Because of the need for skillful microdissection and the time involved in the preparation of primary cultures from tick viscera such cultures have not found widespread use. Development of techniques that permit rapid preparation of long-lived primary cultures from tick hemocytes will make the tick tissue culture system more widely available. Although hemocyte cultures are not as broadly receptive to arboviruses as tissue cultures prepared from tick organs and tissues, such selectivity may, in fact, aid in determining the vector potential of a species for a given pathogen and in the classification and identification of newly isolated viruses. For example, Tuleniy virus recently isolated by us from Ixodes uriae in Oregon is serologically closely related to the B-group mosquito-borne viruses, Japanese B encephalitis (JBE) and WN. An answer to the question of whether or not this is an errant mosquito-borne virus fortuitously being harbored by ticks is suggested from reactions of arthropod tissue culture systems to exposure to Tuleniy virus. JBE and WN will readily multiply in insect cell cultures but not in tick tissue cultures. Tuleniy virus, on the other hand, is rapidly lost in these cultures, but multiplies in tissue cultures prepared from tick viscera, indicating that it is truly a tick-borne entity, perhaps an evolutionary antecedent of the B-group mosquito-borne encephalitides. That it fails to grow in Dermacentor andersoni hemocytes may be a reflection of its specificity for Ixodes ticks.

Production of CPE in insect cells by arboviruses is rare. Evidence that two viruses can produce such effects on C. quinquefasciatus cells simplifies the assay of virus growth in these cells.

If interferon or an interference-type phenomenon can be demonstrated in arthropod cells, a primitive evolutionary origin for this form of defensive mechanism will be evident. Such a phenomenon can be utilized in assay of pathogen growth in arthropod cells.

The adaptation of a line of mosquito cells to serum-free medium is a further step toward growth of insect cells in a defined medium. It also provides a tissue culture system for production of large amounts of R. tsutsugamushi, or other rickettsial antigen, devoid of anticomplementarity caused by serum.

Biochemical and physiological studies of ticks delineate factors critical to survival and propagation of pathogenic microorganisms within ticks and critical to survival of ticks themselves. Development of continuous cultures of tick cells will be aided by these analytical studies.

As in the past, we continued to supply upon request insect cell cultures, consultation and information to institutions and investigators outside the Institute.

Proposed course of project:

Characterization of arbovirus growth and studies of interference-like phenomena in insect and tick cells and developmental aspects of arthropod tissue and cell culture will be continued. Support for continuation of the PL-480 (tick biochemistry and physiology) until mid-1972 is anticipated. Facilities and experience in species identification and monitoring of cell cultures will be expanded. Refinement of mammalian tissue cultures as assay tools for this project and as systems for the production of serological reagents for other projects will be continued as time permits.

Honors and Awards:

Dr. C. E. Yunker

By invitation, co-chaired a colloquium on taxonomy and morphology of parasitic mites held at the 2nd International Congress of Parasitology, Washington, D.C., Sept. 1970.

Continued as consultant for the Smithsonian Army project "Ecology and distribution of mammalian ectoparasites, arboviruses and their hosts in Venezuela".

Appointed External Examiner of theses for the Department of Entomology, Faculty of Sciences, Cairo University, Giza, Egypt, U.A.R.

Appointed to Editorial Committee of the Journal of Medical Entomology.

By request, reviewed research manuscripts for the Journal of Parasitology, Journal of Medical Entomology, and Helminthological Society of Washington.

Publications:

Yunker, C. E., Ormsbee, R. A., Cory, J., and Peacock, M. G.: Infection of insect cells with *Coxiella burneti*. *Acta Virol*. 14: 383-392, 1970.

In press:

Yunker, C. E.: Arthropod tissue culture in the study of arboviruses and rickettsiae: A Review. *Current Topics in Microbiol. and Immunol*.

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